

UNIVERSAL
LIBRARY

OU_150413

UNIVERSAL
LIBRARY

OSMANIA UNIVERSITY LIBRARY

Call No.

616.01/L66I

Accession No.

Author

Levine, Max.

Title

Introduction. Bacteriology

1937

This book should be returned on or before the date
last marked below.

**AN INTRODUCTION TO
LABORATORY TECHNIQUE
IN BACTERIOLOGY**



THE MACMILLAN COMPANY
NEW YORK • BOSTON • CHICAGO • DALLAS
ATLANTA • SAN FRANCISCO

MACMILLAN & CO., LIMITED
LONDON • BOMBAY • CALCUTTA
MELBOURNE

THE MACMILLAN COMPANY
OF CANADA, LIMITED
TORONTO

AN INTRODUCTION TO LABORATORY TECHNIQUE IN BACTERIOLOGY

BY

MAX LEVINE, PH.D.

PROFESSOR OF SANITARY AND TECHNICAL BACTERIOLOGY,

IOWA STATE COLLEGE

BACTERIOLOGIST, IOWA ENGINEERING EXPERIMENT STATION,

AMES, IOWA

Revised Edition

New York

THE MACMILLAN COMPANY

1937

REVISED EDITION COPYRIGHTED, 1933,

By THE MACMILLAN COMPANY

ALL RIGHTS RESERVED—NO PART OF THIS BOOK MAY BE REPRODUCED IN ANY FORM WITHOUT PERMISSION IN WRITING FROM THE PUBLISHER, EXCEPT BY A REVIEWER WHO WISHES TO QUOTE BRIEF PASSAGES IN CONNECTION WITH A REVIEW WRITTEN FOR INCLUSION IN MAGAZINE OR NEWSPAPER.

Printed in the United States of America

Set up and electrotyped. Published August, 1933
reprinted May, 1934; February, 1935; January, 1936;
January, 1937; May, 1937.

First edition copyrighted, 1927,
By The Macmillan Company.
First edition published March, 1927.

To the Memory of My Father

JULIUS B. LEVINE

and Brother

SAMUEL A. LEVINE

PREFACE TO SECOND EDITION

The general form of the exercises adopted for the earlier edition is retained throughout. Changes which experience in handling elementary classes has indicated to be desirable, are introduced in a number of fundamental exercises.

The genus *Bacterium* is now generally subdivided into several genera. In compliance with this newer practice the generic terms *Escherichia*, *Salmonella*, *Proteus*, *Eberthella*, *Shigella*, and *Alcaligenes* are employed.

Exercises of particular interest in the fields of water and food bacteriology are added to supplement the fundamental experiments; the appendices on staining solutions, preparation of culture media, and tests for metabolic end products are brought up to date.

A dichotomous key to a number of species of bacteria is included. No pretense to completeness is made as its scope must necessarily be limited to the use of differential characteristics which the elementary student has employed or may readily utilize. A few pathogenic species as well as the nitrogen fixing, the nitrifying, and acetic acid bacteria are included for the sake of coördination with the classroom and text instruction, but in beginning classes it is suggested that these groups of bacteria be not considered when the key is employed for instructional purposes. Bergey's *Determinative Bacteriology*, Buchanan's *General Bacteriology*, and the author's studies on the intestinal groups of bacteria were drawn upon for descriptions of organisms and the characteristics employed for differentiation.

To Dr. R. E. Buchanan for his continued interest and stimulating enthusiasm for improvement in laboratory instruction the author is very grateful.

MAX LEVINE

AMES, IOWA
July, 1933

PREFACE TO FIRST EDITION

This manual is an outgrowth of a series of laboratory exercises employed for many years by Dr. R. E. Buchanan in the elementary classes in Bacteriology at the Iowa State College. As the title indicates, this is intended merely as an introduction to laboratory technique and does not constitute a comprehensive survey of laboratory methods. Emphasis is placed particularly upon methods and the exercises are designed so as to require a minimum amount of apparatus and only a few organisms. Techniques which are employed in specialized branches of Bacteriology are barely touched upon.

In the preparation of this guide, the more pretentious manuals, particularly Frost's and Giltner's, have been freely consulted, and the author wishes here to express his appreciation for the many valuable suggestions he obtained from them.

To Dr. R. E. Buchanan for many valuable aids and suggestions; to the members of the laboratory staff for giving these exercises a trial in their student laboratories; and to Dr. J. C. Weldin for contributing several of the exercises and reading proof, the author is greatly indebted.

MAX LEVINE

AMES, IOWA
March, 1927

TABLE OF CONTENTS

	PAGE
SUGGESTIONS TO INSTRUCTORS	1
RULES OF THE BACTERIOLOGICAL LABORATORY	5
EXERCISE	
1. USE OF COTTON PLUGS AND STERILIZATION OF GLASS-WARE	9
2. PREPARATION OF NUTRIENT BROTH	11
3. PREPARATION OF NUTRIENT AGAR	13
4. PREPARATION OF BOUILLON GELATIN	15
5. PREPARATION OF MEDIA CONTAINING CARBOHYDRATES, ALCOHOLS, ORGANIC ACIDS	17
6. ADJUSTMENT OF THE REACTION OF A CULTURE MEDIUM	21
7. THE H^+ ION CONCENTRATION OF A MEDIUM	23
8. H^+ ION INDICATORS	27
9. PREPARATION OF HYDROGEN ION STANDARDS.	28
10. REACTION OF CULTURE MEDIA (H^+ ION)	32
11. MORPHOLOGY AND STAINING OF BACTERIA	35
12. THE GRAM STAIN.	38
13. SPORE STAIN	41
14. STAINING METACHROMATIC GRANULES.	44
15. CAPSULE STAIN	46
16. THE ACID FAST STAIN	47
17. MEASUREMENT OF MICROORGANISMS	49
18. MOTILITY OF BACTERIA	51
19. MOTILITY OF BACTERIA IN SOLID MEDIA	53
20. MOTILITY IN SEWAGE AND HAY INFUSION.	54
21. MICROSCOPIC EXAMINATION OF COMPRESSED YEAST AND YEAST FOAM	55
22. MORPHOLOGY OF YOUNG ACTIVELY GROWING YEASTS .	56
23. DISTRIBUTION AND ISOLATION OF YEASTS.	57
24. STUDY OF YEAST SPORES	59

TABLE OF CONTENTS

EXERCISE	PAGE
25. YEAST SPORE FORMATION ON CARROT AGAR	61
26. MORPHOLOGY OF MOLDS.	62
27. STUDY OF MOLDS FROM AIR.	64
28. STUDY OF MOLDS ON DECAYED FOODS.	66
29. IDENTIFICATION OF MOLDS FROM FOODS	67
30. STUDY OF CULTURAL CHARACTERS OF BACTERIA	68
31. STUDY OF CULTURAL CHARACTERS OF YEAST	74
32. ANAEROBIC CULTURE METHODS	75
33. ISOLATION OF PURE CULTURES AND STUDY OF COLONY CHARACTERISTICS OF ESCH. COLI AND B. SUBTILIS ON AGAR PLATES	77
34. ACID AND GAS PRODUCTION FROM GLUCOSE IN LIQUID MEDIA	81
35. ACID AND GAS FORMATION IN SOLID MEDIA	84
36. GAS FORMATION BY YEASTS	86
37. DETECTION OF SPORE FORMERS.	87
38. INDOL PRODUCTION BY BACTERIA	88
39. REDUCTION OF NITRATES TO NITRITES.	90
40. PRODUCTION OF HYDROGEN SULPHIDE	91
41. ACETIC FERMENTATION	92
42. OXALIC FERMENTATION	93
43. LACTIC FERMENTATION AND ACTION OF BACTERIA ON MILK	94
44. BUTYRIC FERMENTATION.	96
45. PANARY FERMENTATION	97
46. ACETYL-METHYL-CARBINOL PRODUCTION	98
47. TYPES OF MICROORGANISMS IN VARIOUS FOOD PROD- UCTS	99
48. COMPARISON OF ACID AND RENNET CURDS	100
49. EFFECT OF STERILIZATION, PASTEURIZATION, AND STOR- AGE TEMPERATURE ON THE KEEPING QUALITIES OF MILK	101
50. DECOMPOSITION OF STARCH BY MICROORGANISMS . .	103
51. DECOMPOSITION OF SUCROSE BY MICROORGANISMS . .	105

TABLE OF CONTENTS

xi

EXERCISE	PAGE
52. DECOMPOSITION OF GELATIN BY MICROORGANISMS . . .	106
53. TO ILLUSTRATE THE RÔLE OF YEAST AND DIASTASE IN BREAD MAKING	108
54. EFFECT OF DIRECT SUNLIGHT ON BACTERIA	109
55. EFFECT OF REACTION ON GROWTH OF MICROORGANISMS	111
56. EFFECT OF DESSICCATION ON BACTERIA	113
57. TO DEMONSTRATE PROPERTIES OF AN ANTISEPTIC AND DISINFECTANT	115
58. ANTISEPTIC ACTION OF VARIOUS MATERIALS	116
59. SELECTIVE ACTION OF GENTIAN VIOLET ON GROWTH OF BACTERIA	117
60. OSMOTIC PRESSURE AND FOOD PRESERVATION . . .	118
61. TO ILLUSTRATE DISINFECTING ACTION OF METABOLIC END PRODUCTS (ACIDS)	120
62. THERMAL DEATH POINT	121
63. EFFECT OF REACTION ON FOOD PRESERVATION . . .	123
64. BACTERIA OF THE AIR	125
65. STUDY OF PIGMENT FORMATION	126
66. THE DESCRIPTIVE CHART FOR THE IDENTIFICATION OF BACTERIA	127
67. QUANTITATIVE BACTERIAL ESTIMATION BY DILUTION .	156
68. QUANTITATIVE BACTERIAL ESTIMATION BY PLATING .	159
69. EXAMINATION OF SURFACE WATER.	161
70. ANALYSIS OF WELL WATER	163
71. EXAMINATION OF MILK AND DETECTION OF ACID FORMING BACTERIA	165
72. THE COLON TYPHOID OR INTESTINAL GROUP OF BAC- TERIA	167
73. DIFFERENTIATION OF INTESTINAL BACTERIA ON SOLID MEDIA	169
74. THE NON-SPECIFIC, PYOGENIC COCCI	170
75. ANIMAL INOCULATION	172
76. PREPARATION OF A VACCINE	173
77. THE MICROSCOPIC (WIDAL) AGGLUTINATION TEST .	176
78. THE MACROSCOPIC AGGLUTINATION TEST.	178

SUPPLEMENTARY EXERCISES IN SANITARY AND FOOD BACTERIOLOGY

EXERCISE	PAGE
79. MICROORGANISMS RESPONSIBLE FOR TASTES AND ODORS IN WATER	183
80. MICROSCOPIC ORGANISMS IN POOLS, LAKES, MUD, RES- ERVOIRS, ETC.	185
81. QUANTITATIVE MICROSCOPIC ANALYSIS OF WATER . . .	186
82. RELATION OF MEDIUM AND TEMPERATURE OF INCUBA- TION TO BACTERIAL COUNT	191
83. DIRECT ISOLATION OF ORGANISMS OF THE COLON GROUP BY PLATING ON SOLID MEDIA	192
84. PRESUMPTIVE TESTS FOR THE COLON GROUP IN WATER	194
85. ISOLATION OF COLON GROUP AFTER PRELIMINARY EN- RICHMENT	196
86. CONFIRMATORY TESTS FOR COLON GROUP OF BACTERIA.	198
87. DIFFERENTIATION OF MEMBERS OF THE COLON GROUP ON SOLID MEDIA	199
88. DIFFERENTIATION AND CLASSIFICATION OF THE COLON GROUP ON THE BASIS OF BIOCHEMICAL REACTIONS . .	201
89. BACTERIOLOGICAL EXAMINATION OF WATER	204
90. DETECTION AND ESTIMATION OF SPORE FORMING LACTOSE-FERMENTING BACTERIA	206
91. DETECTION OF SEWAGE STREPTOCOCCI	208
92. BACTERIOLOGICAL EXAMINATION OF OYSTERS REMOVED FROM THE SHELL	209
93. PHENOL COEFFICIENT	212
94. STANDARDIZATION OF DISINFECTANTS BY F.D.A. METHOD	216
95. MICROANALYSIS OF TOMATO PRODUCTS	219
96. INFLUENCE OF COMPOSITION OF FOOD, AND NATURE OF CONTAMINATION, ON DECOMPOSITION PRODUCTS . .	224
97. BACTERIA IN CANNED FRUITS AND VEGETABLES . . .	226
98. BACTERIA IN WHOLE EGGS	228
99. GROWTH OF BACTERIA IN EGG WHITE	230
100. BACTERIAL CHANGES IN EGGS	231

TABLE OF CONTENTS

xiii

EXERCISE	PAGE
101. BACTERIA IN FROZEN EGGS	232
102. BACTERIAL CONTENT OF HAMBURGER STEAK OR SAUSAGE	234
103. DETECTION OF SPORE FORMING ANAEROBES IN SOIL AND FOODS	236
104. SEPARATION OF SPORE FORMING ANAEROBES FROM SPORE FORMING AEROBES	238
105. SOME DIFFERENTIAL CHARACTERISTICS OF FOOD POISON- ING AND CLOSELY RELATED BACTERIA	239
106. DETECTION OF FOOD POISONING AND TYPHOID- DYSENTERY GROUP OF BACTERIA IN SUSPECTED FOODS OR FECES	241
107. MACROSCOPIC COLONY COUNT OF MILK (A.P.H.A.) (PLATE COUNT)	243
108. METHYLENE BLUE REDUCTION TEST IN MILK	244
109. MICROSCOPIC COLONY COUNT OF MILK	245
110. MICROSCOPIC COUNT OF BACTERIA IN MILK	247
111. DETECTION AND ESTIMATION OF COLON GROUP IN MILK	249
112. EXAMINATION OF FLOUR FOR ORGANISMS RESPONSIBLE FOR ROPY BREAD	250
113. BACTERIOLOGICAL EXAMINATION OF FLOUR	251
114. BACTERIOLOGICAL EXAMINATION OF SUGAR FOR THER- MOPHILIC-CANNED-FOODS-SPOILAGE-ORGANISMS	252

APPENDICES

A. PREPARATION OF STANDARD AND SPECIAL CULTURE MEDIA	257
B. PREPARATION OF STAINING SOLUTIONS AND SPECIAL STAINING METHODS	268
C. TESTS AND REAGENTS FOR METABOLIC PRODUCTS	280
D. GLOSSARY OF DESCRIPTIVE TERMS	286

AN INTRODUCTION TO LABORATORY TECHNIQUE IN BACTERIOLOGY

SUGGESTIONS TO INSTRUCTORS

In devising the following exercises it was the aim to utilize a minimum amount of equipment and to employ only a few organisms. Practically all of the exercises may be performed by the use of the following microorganisms: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus lactis*, and *Serratia marcescens* (*Erythrobacillus prodigiosus*), together with several yeasts and molds. In addition it is considered highly desirable to have some acid fast bacillus such as *Mycobacterium smegmatis* (or *Mycobacterium tuberculosis* in tubercular sputum), and an organism showing metachromatic granules, as *Corynebacterium diphtheriae* in order to acquaint the student with the simpler techniques employed in laboratory diagnosis of disease.

The following organisms are only occasionally employed: *Alcaligenes viscosum*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Lactobacillus bulgaricus* or *Lactobacillus acidophilus*, *Aerobacter cloacae*, *Salmonella cholerae suis*, *Salmonella pullorum*, *Eberthella typhi*, *Salmonella schotmülleri*, *Pseudomonas fluorescens*, *Clostridium sporogenes*, *Sarcina lutea*.

It has been the experience of this laboratory that it is not economical for the elementary student to prepare his own culture media or to be provided with too extensive an assortment of equipment. Each student prepares one batch of nutrient broth and agar or gelatin but thereafter is provided with the necessary culture media (in exchange for clean tubes) and special apparatus as required for individual exercises.

The following has been found to be the practical minimum which each student should have in his locker in order to do satisfactory work in the introductory exercises. Additional equipment will be required for the supplementary exercises on sanitary and food bacteriology.

1 Platinum needle.	1 Box matches.
1 Wire basket.	1 Towel.
20 Microscope slides.	1 Yard cheese cloth.
1 Slide box.	1 Test tube holder.
1 Test tube brush.	1 Slide forceps.
3 Tin cans or other containers for test tubes.	1 Glass writing pencil.
1 Sheet filter paper.	1 Glass rod.
1 Sheet lens paper.	10 Coverslips.

A microscope should be available for each student in a section or laboratory class and sufficient bottles of necessary staining solution should be easily accessible.

Additional equipment may be checked out from the dispensary to be returned immediately on completion of the exercise for which it was required. This plan requires less total equipment and reduces breakage by lessening the crowding of lockers with equipment seldom used.

The following equipment (exclusive of culture media) should be available in the dispensary for each student in a laboratory section:

1 500 c.c. graduate.	2 Ten c.c. pipettes.
1 Glass funnel.	1 Pipette case.
1 Burette.	6 Cork stoppers for test tubes.
2 Evaporating dishes.	4 Smith Fermentation tubes.
1 Stage micrometer.	1 Frost gasometer.
1 Sauce pan.	1 Test tube rack or block.
2 Wire baskets.	1 Ocular micrometer.
1 Bunsen burner.	3 Hollow ground slides.
1 Tripod.	5 Test tubes 200 x 25 mm.
1 Burette clamp.	10 Petri dishes.
1 Ring stand and ring.	6 Large coverslips.
1 Piece cotton flannel.	1 110° C. thermometer.
1 Asbestos pad.	2 Erlenmeyer flasks 100 c.c.
1 Comparator block.	1 Bent glass rod.
10 One c.c. pipettes.	12 Glass beads.

12 Small test tubes 50 to 75 x 5
to 8 mm. for use in Dur-
ham fermentation tubes.

1 Water bath.

1 Box labels.

2 Water sample bottles.

1 Scalpel.

1 Forceps.

2 Capillary pipettes.

2 Ft. glass tubing $\frac{1}{4}$ ".

1 Rubber bulb for capillary
pipettes.

10 Agglutination tubes.

1 Rack for agglutination tubes.

The number of exercises is far in excess of what is expected any student will perform. This enables the instructor to vary the selection of experiments for different sections or school terms. Many of the exercises are designed to meet the vocational objectives of certain groups (as e.g., Home Economics girls) and need not be considered for other students.

The exercises may be divided into two groups: (a) basic, and (b) optional. By a basic exercise is meant one which is required to be performed as distinguished from the optional exercises which are left to the choice of the student. The basic exercises may in turn be divided into two divisions: (1) those considered fundamental for all students, and (2) those which are of particular significance to particular classes (as e.g., Agriculture, Home Economics, etc.).

The basic requirement should be such as to enable the superior student to complete the work in about two-thirds to four-fifths of the allotted time, thus giving him an opportunity to do optional exercises for additional credit. Optional work should not be permitted unless the basic requirements are of a high degree of excellence so as to discourage speed at the expense of thoroughness. The following exercises are considered basic for all students taking 72 laboratory hours per term or semester: 1, 2, 3, 11, 12, 13, 14, 15, 16, 18, 21, 22, 26, 27, 30, 32, 33, 34, 35, 36, 38, 39, 40, 43, 52, 57, 58, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73.

The necessary pure cultures may be obtained from the American Type Culture Collection, 637 South Wood St., Chicago, Illinois.

Descriptive charts of the American Society of Bacteriologists for recording the characteristics of microorganisms

may be purchased from Dr. H. J. Conn, Agricultural Experiment Station, Geneva, New York.

With small classes, the use of prepared dehydrated media (for list of such media see Appendix A) will be found convenient and economical.

RULES OF THE BACTERIOLOGICAL LABORATORY

1. Read over all directions carefully and make certain that they are understood before beginning an exercise. Usually more than one period is required for the completion of an exercise as time must be allowed for the growth and development of the organisms. Plan your work in advance so that if cultures of a certain age are specified they will be on hand when needed.
2. Keep complete notes on each exercise. Answer all questions asked. Make drawings wherever required or wherever they will prove of value. Use a hard lead pencil for drawings.
3. Scrupulous cleanliness of the hands, desk, and all apparatus is absolutely essential, and after working with pathogenic bacteria the tables and hands should be washed with a disinfecting solution.
4. When using the steam sterilizer (autoclave) see that all air has been expelled before the pressure is allowed to rise. The pressure must be raised and, particularly, lowered slowly. Moistening labels and pencils with the tongue, or chewing and eating in the laboratory, is strictly forbidden as a precaution against accidental infection. Infraction of this rule will be considered sufficient to bar a student from the laboratory.
6. If infectious material is accidentally dropped, or in some other way reaches the hands or furniture, an instructor should immediately be notified and the infected area adequately disinfected.
7. Special care should be taken to see that the oil immersion lens is properly wiped with lens paper, immediately after use, and that the microscope is clean before it is returned to its case.

EXERCISES

EXERCISE 1

USE OF COTTON PLUGS AND STERILIZATION OF GLASSWARE

All test tubes and flasks (occasionally pipettes) must be plugged with cotton before sterilization.

The cotton plug: The function of the plug is to prevent the entrance or exit of microorganisms. Dry cotton is admirably adapted to this purpose. It presents a practically perfect barrier to microorganisms, which become enmeshed in the fibers, but air can circulate freely. Bacteria and other forms can grow through plugs that have become wet and to minimize this danger non-absorbent cotton is used.

A. Preparation of Test Tubes and Flasks:

Take a small piece of cotton and by means of a small glass rod insert it into the mouth of the test tube. The plug should project into the tube to a distance of one and one-half inches and sufficient cotton should project out from the mouth to protect the outer edge of the tube. The plug should be tight enough to support the weight of the tube but sufficiently loose to permit its easy removal and replacement. Prepare all test tubes as described.

More stable plugs may be obtained by rolling the cotton.

The method should be demonstrated by the instructor.

Flasks are prepared in the same manner as test tubes but more cotton should be used so that the plug will project two or more inches into the neck of the flask.

B. Sterilization:

The methods of sterilization which you will employ are: flame, dry heat, moist heat, and moist heat under pressure.

1. Place all plugged tubes and flasks in the dry sterilizer at 150°C . for one-half hour. This does not effectively sterilize but will set the plugs.
2. Read in textbook about the construction of the Arnold sterilizer and autoclave. The instructor will explain the principles and methods of operation. The steam sterilizer is applicable for sterilization of water, syringes, surgical dressings, culture media, and other materials which cannot be sterilized in the dry oven. When steam is used under pressure, it is usually sufficient to expose for 15 minutes at 15 pounds (120°C .) but occasionally (as with potatoes and some waters) it is necessary to extend the period to 20 minutes or longer. Sterilization in the Arnold is employed for substances which are subject to decomposition on exposure to high temperatures. In Arnold sterilization the spores are not destroyed and it is therefore necessary to repeat on three or more successive days. In the intervals between sterilizations the spores may germinate, thus forming vegetative cells which are very easily destroyed at 100°C . It is evident that in dealing with an organism which germinates and again forms spores in less than 24 hours, or one whose spores do not germinate for several days, this intermittent sterilization may not be effective.

C. Record and Report:

1. Discuss briefly the origin of the use of cotton plugs and sterilization of glassware.
2. Plot the relation of pressure and temperature for the autoclave.
3. Under what conditions is it necessary to employ:
(a) the dry sterilizer? (b) the Arnold sterilizer?
4. What special precautions must be taken in the operation of the autoclave?
5. Make a diagrammatic sketch of the Arnold sterilizer, showing path of steam by arrows.

EXERCISE 2

PREPARATION OF NUTRIENT BROTH

In the preparation of media students will be permitted to work in pairs but each student will be held responsible for a knowledge of the ingredients and preparation of all ordinary media.

Non-official Laboratory Method

(See Appendix for Official Method)

A. Procedure:

1. Secure from the dispensary:

3 grams beef extract.
5 grams peptone.
5 grams NaCl.

2. Place in saucepan with 1 liter of water.
3. Weigh.
4. Bring to a boil over the gas flame and boil for 5 minutes.
5. Make neutral to phenolphthalein. This may be done by adding small quantities (5 c.c.) of normal NaOH and after each addition test a drop of the medium on a piece of phenolphthalein paper. Continue addition of NaOH until a faint pink is obtained when tested as indicated. The paper should not be placed in the medium.
6. Make up the loss due to evaporation, add 25 to 50 c.c. of water in excess and boil down to weight.
7. Filter through absorbent cotton and cotton flannel until clear.
8. Fill 10 tubes about one-third full and place the remainder in flasks.
9. Sterilize in the autoclave at 15 pounds for 15 minutes.

B. Record and Report:

1. In what respect does this method of the preparation of broth differ from the standard method?
2. (a) What is infusion broth; (b) peptone; (c) beef extract?

Ref.: Buchanan. *Bacteriology*, p. 117.

EXERCISE 3

PREPARATION OF NUTRIENT AGAR

Nutrient agar is the most commonly employed solid medium for the growth of bacteria and consists of nutrient broth to which agar-agar has been added for the purpose of gelatinizing. Agar-agar is a seaweed found near the Uduan Indian Ocean in the Chinese and Japanese waters. It is a nitrogen free carbon compound but may contain a minute quantity of nitrogen and inorganic materials as impurities. Its colloidal properties are not affected appreciably by sterilization or the action of most microorganisms but the reaction of the medium exerts a considerable influence on its gelatinizing ability. If the medium is too acid the agar-agar will be hydrolized on sterilization. When it is necessary to use agar of high acidity, it is essential that the acid be added after the medium has been prepared and sterilized, and just before it solidifies.

Non-official Laboratory Method

(See Appendix for Official Method)

A. Procedure:

1. Prepare a liter of nutrient broth as described in Exercise 2 (or the broth previously prepared may be used for this experiment).
2. Weigh the broth and add $1\frac{1}{2}\%$, by weight, of agar-agar.
3. Boil over the free flame until thoroughly dissolved. This may take from 20 to 30 minutes.
4. Neutralize, if necessary; make up the loss due to evaporation; add 25 to 50 c.c. of water in excess and boil down to weight.
5. Filter through absorbent cotton and cotton flannel.

Note: The agar must be kept hot while filtering and to facilitate the process, boiling water should be passed through the filter just before the agar. If a new piece of cotton flannel is used it is important that it be thoroughly washed to assist in the filtration.

6. Fill ten tubes one-fourth full and ten tubes one-half full. Place the remainder in a flask.
7. Sterilize in the autoclave for 15 minutes at 15 pounds.
8. Place the tubes one-fourth full in a slanting position to cool, so as to make a slope about 2 inches long.

B. Record and Report:

1. What is agar-agar?
2. At what approximate temperature does agar (a) solidify; (b) liquefy?
3. What products are formed on hydrolysis of agar-agar?

Ref.: Buchanan. *Bacteriology*, pp. 118-120.

EXERCISE 4

PREPARATION OF BOUILLON GELATIN

Bouillon gelatin was the first solidifiable medium employed in bacteriology. It was introduced in 1882 by Dr. Robert Koch of Germany. This revolutionized the science.

Gelatin is a protein and differs fundamentally from agar-agar in that it is relatively easily attacked and hydrolized by heat and by many microorganisms.

In a concentration of 10 to 15%, gelatin melts or liquefies at about 25° C. It is therefore not applicable as a solid medium for the cultivation of bacteria which require temperatures above this for their growth. Overheating in the process of sterilization and preparation will also tend to lower the melting point of gelatin and it is not an uncommon occurrence that the product will stay liquid at room temperature. Too much emphasis cannot therefore be put upon the necessity for adhering carefully to directions. Never heat the gelatin over a free flame but employ an asbestos pad to avoid burning as gelatin has a tendency to stick to the bottom. If the medium does not solidify it will be necessary to repeat this exercise until one is obtained which will remain solid at room temperature.

Non-official Laboratory Method

(See Appendix for Official Method)

A. Procedure:

1. Prepare a liter of broth as in Exercise 2.
2. To 300 c.c. add 12 to 15% (36-45 g.) gelatin; weigh.
3. Heat over an asbestos pad, stirring the gelatin until it is dissolved. Continue heating for five minutes; the temperature should not rise above 65° C.

4. Neutralize as described in Exercise 2. Make up loss due to evaporation. Heat at 65° C. for 15 minutes.
5. Filter through absorbent cotton and cotton flannel until clear. The filter should be washed first with boiling water.
6. Fill ten tubes one-half full.
7. Sterilize in the autoclave for 15 minutes at 15 pounds.
8. Immediately after sterilization place tubes in cold water. This will assist in solidification.

B. Record and Report:

1. How does gelatin differ from agar-agar with regard to:
 - a. melting point?
 - b. effect of sterilization on solidification?
 - c. chemical composition?
 - d. availability for bacterial nutrition?
 - e. hydrolytic products?
2. What is the source of gelatin?

Ref.: Buchanan. *Bacteriology*, pp. 118-119.

EXERCISE 5

PREPARATION OF MEDIA CONTAINING CARBOHYDRATES, ALCOHOLS, ORGANIC ACIDS

Nutrient solutions containing various pure carbon compounds are often employed in bacteriology to detect the fermentative action of different kinds of microorganisms. Among the more important and most commonly employed substances are the following:

Monosaccharids

Pentoses

Arabinose $(C_5H_{10}O_5)$

Xylose $(C_5H_{10}O_5)$

Methyl Pentose

Rhamnose $(C_6H_{12}O_5)$

Hexoses

Glucose $(C_6H_{12}O_6)$

Levulose $(C_6H_{12}O_6)$

Galactose $(C_6H_{12}O_6)$

Mannose $(C_6H_{12}O_6)$

Disaccharids

Reducing Sugars

Lactose $(C_{12}H_{22}O_{11})$

Maltose $(C_{12}H_{22}O_{11})$

Non-reducing Sugars

Sucrose $(C_{12}H_{22}O_{11})$

Trehalose $(C_{12}H_{22}O_{11})$

Trisaccharids

Raffinose $(C_{18}H_{32}O_{16})$

Melezitose $(C_{18}H_{32}O_{16})$

Glucodises

Amygdalin	(C ₂₀ H ₂₇ NO ₁₁)
Salicin	(C ₁₃ H ₁₈ O ₇)
Aesculin	(C ₁₅ H ₁₆ O ₉)

Alcohols*Monohydric*

Methyl	(CH ₃ OH)
Ethyl	(C ₂ H ₅ OH)
Iso-propyl	(C ₃ H ₇ OH)
N-butyl	(C ₄ H ₉ OH)
Iso-butyl	(C ₄ H ₉ OH)
Iso-amyl	(C ₅ H ₁₁ OH)

Trihydric alcohol

Glycerol	(C ₃ H ₅ (OH) ₃)
----------	--

Tetrahydric alcohol

Erythritol	(C ₄ H ₆ (OH) ₄)
------------	--

Pentahydric alcohol

Adonitol	(C ₅ H ₇ (OH) ₅)
Arabitol	(C ₅ H ₇ (OH) ₅)

Hexahydric alcohols

Mannitol	(C ₆ H ₈ (OH) ₆)
Sorbitol	(C ₆ H ₈ (OH) ₆)
Dulcitol	(C ₆ H ₈ (OH) ₆)

Heptahydric alcohol

Perseitol	(C ₇ H ₉ (OH) ₇)
-----------	--

Hydroxy derivative of hexahydrobenzene

Inositol	(C ₆ H ₆ (OH) ₆)
----------	--

Aldehydes

Benzaldehyde	(C ₆ H ₅ CHO)
--------------	-------------------------------------

Acids*Fatty acids*

Formic	(HCOOH)
Acetic	(CH ₃ COOH)
Propionic	(C ₂ H ₅ COOH)
Butyric	(C ₃ H ₇ COOH)
Caproic	(C ₅ H ₁₁ COOH)
Stearic	(C ₁₇ H ₃₅ COOH)

*Amino acids*Glycocoll $(\text{CH}_2\text{NH}_2\text{COOH})$ Leucine $(\text{C}_4\text{H}_9\text{CHNH}_2\text{COOH})$ Tryptophane $(\text{C}_8\text{H}_6\text{NC}_2\text{H}_3\text{NH}_2\text{COOH})$ Asparagine $(\text{NH}_2\text{OCCH}_2\text{CHNH}_2\text{COOH})$ *Aldehydic acid*Glyoxylic (CHOCOOH) *Halogen acid*Chloracetic $(\text{CH}_2\text{ClCOOH})$ *Polyhydric acids*Gluconic $(\text{C}_5\text{H}_6(\text{OH})_5\text{COOH})$

(as calcium gluconate)

Chinic $(\text{C}_6\text{H}_7(\text{OH})_4\text{COOH})$

(as calcium chinate)

*Dibasic acids*Oxalic $(\text{COOH})_2$ Succinic $(\text{COOHCH}_2)_2$ *Hydroxy-dibasic acids*Malic $(\text{COOHCHOHCH}_2\text{COOH})$ Tartaric $(\text{COOHCH}_2\text{OH})_2$ *Hydroxy-tribasic acid*Citric $\text{CH}_2\text{COHCH}_2(\text{COOH})_3$ *Ureides*Uric acid $(\text{C}_5\text{H}_4\text{O}_3\text{N}_4)$ *Aromatic acids*Benzoic $(\text{C}_6\text{H}_5\text{COOH})$ Salicylic $(\text{C}_6\text{H}_4\text{OHCOOH})$ Cinnamic $(\text{C}_6\text{H}_5\text{CH:CHCOOH})$ **A. Materials Needed:**

- | | |
|---|------------|
| 1. Peptone..... | 10 g. |
| 2. Dipotassium phosphate (K_2HPO_4) | 2 g. |
| 3. Water (distilled)..... | 1000 c.c. |
| 4. Carbon compound..... | 3 to 10 g. |

B. Procedure:

1. Dissolve the peptone and dipotassium phosphate in the water, bring to a boil and add from 0.3 to 1.0% of

the desired carbohydrate. Adjustment of the reaction is generally not necessary.

Note: Dunham's solution (see Appendix A) or neutral nutrient broth may be employed in place of the peptone phosphate base.

2. Place in fermentation tubes and sterilize in the Arnold by the intermittent method for three successive days. Sterilization may preferably be performed in the autoclave at 10 pounds for 10 minutes but it is desirable that the media should be cooled just as rapidly as possible. Over-sterilization should be avoided to prevent hydrolysis and caramelization of the carbohydrates.

C. Record and Report:

1. Define or explain:
 - a. monosaccharid.
 - b. polysaccharid.
 - c. glucoside.
 - d. polyatomic alcohol.
 - e. pentose.

EXERCISE 6

ADJUSTMENT OF THE REACTION OF A CULTURE MEDIUM

(Total Acidity; Phenolphthalein Titration)

The reaction of media is very important as different types of bacteria have different optimum reactions for maximum growth. The method to be described below is perhaps still frequently employed. It is well to bear in mind that the titration obtained is not indicative of the true acidity or alkalinity of the medium but when working with the same constituents, an investigator will be able to obtain comparable media. (See next exercise on H^+ ion concentration.)

This titration method for determining the acidity of a medium is particularly valuable in ascertaining the total amount of acid produced in many industrial processes as in vinegar production and the dairy industry.

A. Materials Needed:

1. N/20 NaOH.
2. Burette.
3. Phenolphthalein indicator.
4. Evaporating dish or casserole.
5. Broth or agar (200 to 500 c.c.) in a flask.

B. Procedure:

1. Place 45 c.c. of distilled water in a casserole or evaporating dish.
2. Add 5 c.c. of the medium to be tested.
3. Add 0.5 c.c. phenolphthalein indicator.
4. Boil for one minute to expel CO_2 .
5. Titrate while hot with N/20 NaOH. A faint rose color which is permanent for at least one minute should be taken for the end point.

Note: In titrating 5 c.c. with N/20 alkali as above, every 1/10 of a c.c. of alkali used corresponds to one degree of Fuller's scale. Every c.c. employed is a direct measure of the per cent normality of the medium, e.g., if it takes 3.5 c.c. of N/20 NaOH to neutralize 5 c.c. of the culture medium, then it will require 3.5 c.c. of N/1 NaOH to neutralize the acid in 100 c.c. of the medium tested. We say, therefore, that the medium is 3.5% N/1 (normal) acid and indicate it thus: +3.5.

6. Adjustment of reaction:

- a. To change the reaction to any desired point, say to +1 in the example given above, it would be necessary to add 2.5 c.c. of N/1 alkali to 100 c.c. of the medium.
- b. Adjust the reaction of the medium which you examined to +1 by the addition of normal acid or alkali and retitrate to ascertain the success of the adjustment.

C. Record and Report:

1. Record the reaction in degrees of Fuller's scale, which is the number of c.c. of normal (N/1) acid or alkali present in 1000 c.c. of the medium.
2. Reaction of medium furnished
N/1 NaOH used for adjustment to +1.....c.c.
Reaction after adjustment
3. It required 4.5 c.c. of N/20 NaOH to neutralize 5 c.c. of broth. Assuming that the acidity is due to lactic acid, what is the concentration of lactic acid in grams per liter?
4. A batch of agar titrates +2.8. How much normal NaOH is required to bring the reaction of 800 c.c. of the medium to +1; to -1.50?

EXERCISE 7

THE H^+ ION CONCENTRATION OF A MEDIUM

A. Discussion:

Normality and H^+ ion concentration: In the previous exercise the reaction of the medium prepared was adjusted with respect to the concentration of acid or alkali expressed in terms of normality. A normal solution of an acid is defined as one having one gram of replaceable or acid hydrogen per liter. Thus 36.5 grams of hydrochloric acid $\frac{(H-Cl)}{1-35.5}$ or 60 grams of acetic acid $\frac{(H-C_2H_3O_2)}{1-59}$ per liter are both normal solutions, for they each contain 1.0 gram of hydrogen which is capable of combining with the hydroxyl (OH) group of a base. These two acids are, however, very different with respect to their H^+ ion, concentrations. In a normal solution of HCl, 81.0% of the acid is dissociated into H^+ ions, and Cl^- ions so that the actual concentration of free H^+ ions is 0.81 gram per liter. A normal solution of acetic acid is only 0.4% dissociated into H^+ and Ac^- ions so that the concentration of H^+ ions is only 0.004 grams per liter. Thus there are 200 times as many H^+ ions in the N/1 hydrochloric acid as there are in the N/1 acetic acid. It must be apparent therefore that the determination of the normality of a medium is an unreliable index of its H^+ ion concentration.

Different microorganisms require different H^+ ion concentrations for their optimum growth. A culture medium, therefore, should be adjusted to a definite

H^+ ion concentration rather than on an acid normality basis.

H^+ ions and pH: A solution containing one gram of acid hydrogen (i.e., H capable of combining with the OH of a base) constitutes a normal acid solution. Similarly a solution containing one gram of hydrogen in the ionized state, or H^+ ions, is known as a normal H^+ ion solution.

Water dissociates into positive H^+ ions and negative OH^- ions, and in pure distilled water the concentration of H^+ ions has been found, by appropriate electrical measurements, to be 1/10,000,000 grams per liter. To avoid the use of this unwieldy fraction it may be written 10^{-7} . As H^+ ion concentrations are always expressed by an exponent to the base 10, this base need not be indicated, and by common consent the minus sign of the exponent is also dropped; the H^+ ion concentration of pure distilled water is therefore expressed by the figure 7.0 which is known as its pH value.

The pH is really the logarithm of the reciprocal of the H^+ ion concentration in grams per liter. Thus in pure distilled water the concentration of H^+ ions is 1/10,000,000 gram per liter. The reciprocal of 1/10,000,000, is 10,000,000, the common logarithm of which is 7. This is the pH for distilled water.

The relation between H^+ ion concentration and pH is indicated in the following table. It will be noted that

(1) H^+ ION CONCENTRATION IN NORMALITY	(2) H^+ ION CONCENTRATION IN GRAMS PER LITER	(3) pH [LOG. OF RE- CIPROCAL OF (2)]
N/1	1/1 or 10^{-0}	0.0
N/10	1/10 or 10^{-1}	1.0
N/100	1/100 or 10^{-2}	2.0
N/1,000	1/1,000 or 10^{-3}	3.0
N/10,000	1/10,000 or 10^{-4}	4.0
N/100,000	1/100,000 or 10^{-5}	5.0
N/1,000,000	1/1,000,000 or 10^{-6}	6.0
N/10,000,000	1/10,000,000 or 10^{-7}	7.0
N/1,000,000,000	1/1,000,000,000 or 10^{-9}	9.0

as the pH increases the acidity or actual concentration of H^+ ions decreases.

Determination of H^+ ion concentration may be made by electrical measurements, or by the use of indicators. For ordinary routine work, the indicators are sufficiently reliable. An indicator is a substance which, when present in a solution, changes color as the acidity or alkalinity of the solution changes. There are a number of indicators, and they change color at different (but for each indicator at a definite), H^+ ion concentration. Litmus for example is lilac at pH 7.0, becoming red in more acid solutions ($pH < 7.0$) and blue in more alkaline ($pH > 7.0$) solutions. Phenol red is yellow in solutions more acid than pH 6.8 and pink to red in more alkaline solutions. Methyl red is yellow in solutions more alkaline than pH 6.0 and red in more acid solutions.

Indicator range: The student will become familiar with the more important indicators applicable for H^+ ion concentration determinations in the following exercises. It remains to explain what is meant by the indicator range. Phenol red is yellow in H^+ ion concentration more acid than pH 6.8 at which point it changes to a faint pink, becoming progressively more red as the solution becomes more alkaline until pH 8.4 is reached, beyond which there is no further change in color. pH 6.8 to pH 8.4 is the range within which phenol red may be employed for estimating H^+ ion concentrations, for it is only within these limits that the indicator exhibits different recognizable shades of color which correspond to definite H^+ ion concentrations.

A list of the more common H^+ ion indicators is given in the table on the following page.

B. Record and Report:

1. Why is total acidity undesirable as an index of the reaction of a culture medium?

LIST OF INDICATORS (AFTER CLARK AND LUBS)

CHEMICAL NAME	COMMON NAME	CON- CEN- TRATION	COLOR CHANGE Ac.-ALK.	RANGE pH
Thymol sulphon-phthalein (acid range)	Thymol blue	0.04%	Red-yellow	1.2-3.8
Tetra bromo phenol sulphon-phthalein	Brom phenol blue	0.04%	Yellow-blue	3.0-4.6
Tetra bromo meta cresol sulphon-phthalein	Brom cresol green	0.04%	Yellow-green	3.8-5.4
Ortho carboxy benzene azo dimethyl anilin	Methyl red	0.02%	Red-yellow	4.4-6.0
Ortho carboxy benzene azo dipropyl anilin	Propyl red	0.02%	Red-yellow	4.8-6.4
Dibromo cresol sulphon-phthalein	Brom cresol purple	0.04%	Yellow-purple	5.2-6.8
Dibromo thymol sulphon-phthalein	Brom thymol blue	0.04%	Yellow-blue	6.0-7.6
Phenol sulphon-phthalein	Phenol red	0.02%	Yellow-red	6.8-8.4
Ortho cresol sulphon-phthalein	Cresol red	0.02%	Yellow-red	7.2-8.8
Thymol sulphon-phthalein (alkaline range)	Thymol blue *	0.04%	Yellow-blue	8.0-9.6*
Ortho cresol phthalein	Cresol phthalein	0.02%	Colorless-red	8.2-9.8

* Phenolphthalein colorless to red has same range.

2. On a piece of plotting paper indicate graphically the range of color change of the following indicators: cresol red, phenol red, brom thymol blue, brom cresol purple, brom cresol green, methyl red, thymol blue, and phenol blue.

Ref.: Buchanan. *Bacteriology*, pp. 112-116, 139-142.

Manual of Methods. Soc. Am. Bact. Leaflet IX.

EXERCISE 8

H⁺ ION INDICATORS

A. Materials Needed:

- | | |
|-------------------------------------|--|
| 1. Phenol red. ¹ | 6. N/20 NaOH. |
| 2. Brom thymol blue. ¹ | 7. N/20 HCl. |
| 3. Brom cresol purple. ¹ | 8. N/5 HC ₂ H ₃ O ₂ . |
| 4. Methyl red. ¹ | 9. N/10 NH ₄ OH. |
| 5. Thymol blue. ¹ | |

B. Procedure:

1. To a few c c. of N/20 HCl add a few drops of each of the above indicators and record color.
2. Repeat with N/20 NaOH and N/5 HC₂H₃O₂ and N/10 NH₄OH.

C. Examination and Record:

1. Record in tabular form the color reaction of the different indicators with the acids and alkalis employed. Note particularly whether the color produced is acid or alkaline with respect to the indicator.

¹ See Appendix C for method of preparation of indicator solutions.

EXERCISE 9

PREPARATION OF HYDROGEN ION STANDARDS

The standards described below are particularly well adapted to elementary classes in bacteriology. For more delicate standards the student is referred to Clark's book on Hydrogen Ions.

I. SINGLE TUBE STANDARDS ¹

A. Materials Needed:

1. M/1 acetic acid (57.7 c.c. glacial acetic acid diluted to 1000 c.c. with distilled water).
2. M/1 sodium acetate (82.5 grams anhydrous sodium acetate per liter).
3. M/10 KH_2PO_4 (13.62 grams anhydrous primary potassium phosphate per liter).
4. M/10 Na_2HPO_4 (14.21 grams anhydrous secondary sodium phosphate per liter).
5. Indicator solution.
6. Pipettes (1 c.c. and 10 c.c.).

B. Procedure:

1. Standards may be prepared by mixing the solutions indicated in the table below.
- ✓ 2. To 5 c.c. of each standard in a clean tube add 0.5 c.c. (10% by volume) of an appropriate indicator to make working colorimetric standards.

¹ Ref.: Clark, Wm. M. *The Determination of Hydrogen Ion Concentration*. Williams and Wilkins. 1928.

McCrudden, F. H. *The Determination of Hydrogen Ion Concentration*, Reprint 730, U.S.P.H. Reports.

Prepared single tube standards may be obtained from the La Motte Chemical Products Co., Baltimore, Md.

PH VALUE	c.c. $\text{NaC}_2\text{H}_3\text{O}_2$ M/1	c.c. $\text{HC}_2\text{H}_3\text{O}_2$ M/1
4.3	30	70
4.6	50	50
4.8	60	40
5.0	70	30
5.2	80	20
5.4	85	15
5.6	90	10
	c.c. Na_2HPO_4 M/10	c.c. KH_2PO_4 M/10
5.8	8	92
6.0	12	88
6.2	19	81
6.4	27	73
6.6	37	63
6.8	49	51
7.0	61	39
7.2	73	27
7.4	82	18
7.6	89	11
7.8	94	6
8.0	97	3

II. DOUBLE TUBE STANDARDS WITHOUT BUFFER SOLUTIONS ¹

The following set of standards may be readily prepared without the use of carefully standardized chemicals, and are applicable for class and field work.

A. Materials Needed:

1. HCl (approximate 0.001, 0.1, and 0.5%).
2. NaOH (approximate N/20 and N/200).
3. Clean test tubes of uniform size.
4. Indicator solutions.
5. Pipettes (graduated in 0.1 c.c., 1.0 c.c., and 10.0 c.c.).

B. Procedure:

1. Arrange 18 tubes in two rows of 9 tubes.
2. Label each pair of tubes from 1 to 9.

¹ Ref.: Medalia, L. *Jour. of Bact.*, Vol. 5, p. 441 (1920).
 Gillespie, *Journal of Am. Chem. Soc.*, Vol. 42, p. 742 (1920).
Soil Science, Vol. 9, p. 115 (1920).

3. To each tube in one of the rows add 10 c.c. of alkali and to the tubes in the other row add 10 c.c. of acid as indicated in the table below.
4. Add indicator as follows:

PAIR	ALKALI TUBES	ACID TUBES
1	0.0 c.c.	0.8 c.c.
2	0.1 c.c.	0.7 c.c.
3	0.2 c.c.	0.6 c.c.
4	0.3 c.c.	0.5 c.c.
5	0.4 c.c.	0.4 c.c.
6	0.5 c.c.	0.3 c.c.
7	0.6 c.c.	0.2 c.c.
8	0.7 c.c.	0.1 c.c.
9	0.8 c.c.	0.0 c.c.

On viewing the pairs of tubes by transmitted light it will be observed that pair No. 1 gives the full acid color and pair No. 9 the full alkaline color. The other pairs show intermediate colors, each of which corresponds to a definite pH as given in the table below.

To assist in observing the colors of the pairs of tubes it is necessary to employ a comparator block. This consists of a block with vertical holes into which the tubes fit snugly, and smaller horizontal holes or slits (bored through the vertical holes) through which the tubes may be viewed. (See Exercise 10.)

DATA FOR THE PREPARATION OF THE COLOR STANDARDS (after Medalia)

INDICATOR WATERY SOLUTION *		pH VALUES OF EACH PAIR OF TUBES							
Acid Tube	Alk. Tube	Thymol Blue (acid range)	Brom. Phenol Blue	Methyl Red	Brom. Cresol Purple	Brom. Thymol Blue	Phenol Red	Cresol Red	Thymol Blue (alk. range)
c.c.	c.c.	pH	pH	pH	pH	pH	pH	pH	pH
0.8	0	1.2	3.2	4.4	5.2	6.2	6.8	7.2	8.0
0.7	0.1	1.4	3.4	4.7	5.4	6.4	7.0	7.4	8.2
0.6	0.2	1.6	3.6	4.9	5.6	6.6	7.2	7.6	8.4
0.5	0.3	1.8	3.8	5.1	5.9	6.8	7.4	7.8	8.6
0.4	0.4	2.0	4.0	5.2	6.1	7.0	7.6	8.0	8.8
0.3	0.5	2.2	4.2	5.4	6.3	7.2	7.8	8.2	9.0
0.2	0.6	2.4	4.4	5.6	6.5	7.5	8.0	8.4	9.2
0.1	0.7	2.6	4.6	5.8	6.7	7.6	8.2	8.6	9.4
0	0.8	2.8	4.8	6.0	6.8	7.8	8.4	8.8	9.6
Per Cent Indicator									
Water Sol. *		0.02%	0.02%	0.02%	0.02%	0.02%	0.04%	0.02%	0.02%
Sol. to produce alk. color (10 c.c.)		0.001% HCl	N/200 NaOH	N/20 NaOH	N/20 NaOH	N/20 NaOH	N/100 NaOH	N/20 NaOH	N/20 NaOH
Sol. to produce acid color (10 c.c.)		0.5% HCl	0.1% HCl	0.1% HCl	0.1% HCl	0.1% HCl	0.1% HCl	0.1% HCl	0.001% HCl
Color of the "Color Standards"		Red through red-yellow to yellow	Yellow through green to blue	Red through red-yellow to yellow	Yellow through green-pink to purple	Yellow through green to blue	Yellow through pink to red	Yellow through pink to red	Yellow through green to blue

* Made from alcoholic 0.2% stock solution.

EXERCISE 10

REACTION OF CULTURE MEDIA (H^+ ION)

A. Materials Needed:

1. H^+ ion standards.
2. H^+ ion indicators.
3. Clean test tubes.
4. Distilled water.
5. Medium for test.
6. N/20 NaOH.
7. Comparator block.

B. Procedure:

1. Adjustment to neutrality to phenol red.

- a. Withdraw 5 c.c. of the medium, dilute with 5 c.c. of distilled water and add 10 drops of a solution of phenol red (phenol-sulphon-phthalein).
- b. Titrate with a N/20 (or 1/20 of a standard alkali solution) NaOH until the phenol red shows a slight but distinct pink color. Calculate the amount of standard NaOH solution which must be added to the medium to reach this reaction. After the addition check the reaction by adding 10 drops of phenol red to 5 c.c. of the medium diluted with 5 c.c. of water.

2. Adjustment of reaction to desired pH.

- a. To 45 c.c. of distilled water in an evaporating dish, add 5 c.c. of a standard having the desired pH, and 1.0 c.c. of an appropriate indicator. This will give the shade to which the medium must be titrated. (pH 7.0 with brom thymol blue or pH 7.6 with phenol red is suggested as convenient for class use.)

- b. To 45 c.c. of distilled water in another evaporating dish add 5 c.c. of the medium under consideration, and 1 c.c. of the same indicator as in the standard. Titrate with N/20 NaOH or HCl until color matches the standard. Calculate the amount of N/1 NaOH or HCl required per liter of medium to give the desired reaction.

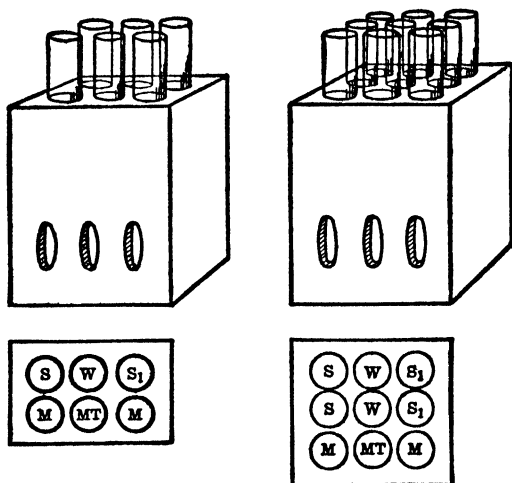


FIG. 1.

TWO-HOLE COMPARATOR BLOCK
FOR SINGLE TUBE STANDARDS.

THREE-HOLE COMPARATOR BLOCK
FOR DOUBLE TUBE STANDARDS.

W, distilled water; M, test material without indicator; MT, test material with indicator; S, H^+ ion standards.

3. Determination of the H^+ ion concentration of a medium.

- a. To 9 c.c. of the unknown medium in a test tube (the medium may be diluted ten times, if desired, to reduce turbidity or color) add 1.0. c.c. of an appropriate indicator (one whose range covers the reaction of the medium).

- b. Place this tube in the front vertical hole of the comparator.
- c. In the two holes behind this tube place two tubes of distilled water.
- d. In the front vertical hole, at the side of the unknown, place a tube containing 10 c.c. of the unknown solution without any indicator (diluted ten times if such a solution was used with the indicator).
- e. In the holes behind this tube insert a pair of standards containing the indicator employed in the unknown, and look through the block at a good source of light (preferably daylight) to see if it matches.
- f. Insert various pairs of standards until the correct pair is found. The H^+ ion concentration may then be read from the tables given above.

Note: If single tube standards are employed a two-hole comparator is used instead of the three-hole block described for the double standards. (See Figure.)

C. Examination and Record:

1. What is meant by neutrality in culture media?
2. What does pH 3.0 designate?
3. Record c.c. N/20 NaOH required to bring mediums furnished in B-1 and B-2 to neutrality and the designated reactions respectively.

Ref.: Buchanan. *Bacteriology*, pp. 112-116.

EXERCISE 11

MORPHOLOGY AND STAINING OF BACTERIA

It is the purpose of this exercise to introduce the student, (1) to the method of preparing smears and staining bacteria, and (2) to acquaint him with the fundamental morphological differences employed for distinguishing different bacterial types.

A. Materials Needed:

1. Agar slant cultures (24 to 48 hours old) of the following:
 - a. *Escherichia (Bacterium) coli*.
 - b. *Bacillus subtilis*.
 - c. *Staphylococcus aureus*.
 - d. *Streptococcus lactis* (a milk culture may be employed).
 - e. *Spirillum rubrum* or other spiral organism.
2. Gentian violet stain.
3. Microscope and slides.
4. Platinum needle.

B. Procedure:

1. Stained mounts of *Esch. (Bact.) coli*.
 - a. Clean carefully a microscope slide by washing thoroughly in soap and water, drying, and heating in the flame to drive off any oil and grease. When clean, a drop of water will spread evenly over the surface of the slide with no tendency to round up into drops. It is essential to the success of this experiment that slides be scrupulously clean.
 - b. Place a drop of water on the slide by means of a standard platinum loop (4 mm. in diameter). Singe the cotton plug and sterilize the platinum needle (by bringing it to red heat) in the flame.

- c. Remove the cotton plug, sterilize the mouth of the tube by passing it through the flame, then take a small quantity of the material from the culture of *Escherichia coli* on the tip of the sterile straight platinum needle, and reinsert the plug in the tube.
- d. Mix this bit of culture (on the end of the needle) with the drop of water on the slide and spread in a thin layer so that about one square inch of the slide is covered.

Caution: Sterilize the platinum needle by heating in the flame immediately after making the smear.

- e. Dry the slide in the air. (The drying may be hastened by holding the slide well above a flame.)
 - f. When completely dry, fix the bacteria onto the slide by passing it through the flame (smear side up) two or three times.
 - g. Flood the smear with gentian violet and stain for fifteen seconds. (The slide may be held in a slide forceps if desired.)
 - h. Wash off the stain in a gentle stream of tap water.
 - i. Dry in the air or over the flame.
 - j. Label, indicating—(1) organism, (2) stain employed, (3) date, (4) culture medium, and (5) student's name or initials.
- 2. Stained mounts of *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus lactis* and the spirillum should be prepared as directed above.
 - 3. Prepare stained mount of the tartar from the teeth as directed under *Escherichia (Bact.) coli*. Tartar may be obtained by scraping the teeth with a sterile platinum loop.

C. Examination and Record:

- 1. Place a drop of immersion oil (cedar) on the mount and examine with the oil immersion lense.

2. Make a sketch showing the appearance of the organisms as seen with the microscope. The cells of *Bacillus subtilis* should be at least one-fourth inch in length and the other organisms should be made of corresponding size.
3. Describe briefly the morphology of the cells, using the terms indicated below. Write description under the sketches.

Form: spherical, short rods, long rods, filaments, commas, short spirals, long spirals, clostridium, cuneate, clavate.

Grouping: single, irregular masses, pairs, tetrads, short chains, long chains, packets.

Ends of rods: rounded, truncate, concave.

Ref.: Buchanan. *Bacteriology*, pp. 21-24, 151-153.

EXERCISE 12

THE GRAM STAIN

The purpose of this exercise is to acquaint the student with a special staining technique which is perhaps the most commonly employed stain for differentiating bacterial species. The student should refer to the appendix for other methods than that given below.

A. Materials Needed:

1. Young cultures of *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* on agar (not more than 24 hours old).
2. Young culture (24 hours) of *Streptococcus lactis* in milk.
3. Staining solutions.
 - a. Anilin oil gentian violet.
 - b. Lugol's solution.¹
 - c. Ninety-five per cent alcohol.
 - d. Dilute saffranin.
 - e. Crystal violet-ammonium oxalate solution (Hucker.)
4. Microscope, slides, needles, flame, etc.

B. Procedure:

1. Gram stain of *Bacillus subtilis*.
 - a. Prepare a smear, dry, and fix as described in Exercise 11 (B-1-a to f).
 - b. Flood the slide with anilin oil gentian violet; allow this stain to act for one and one-half minutes, or flood with crystal violet-ammonium oxalate for one minute.
 - c. Wash in water.
 - d. Apply Lugol's solution to the smear; allow it to remain for one minute.

¹ Sometimes referred to as "Gram's Iodine Solution."

- e. Rinse with alcohol until no color comes off, if the anilin oil gentian violet was used, or for one minute if the crystal violet-oxylate stain was employed.
 - f. Wash in water.
 - g. Stain with dilute saffranin for 15 seconds.
 - h. Wash in water.
 - i. Dry in the air or well over the flame.
 - j. Label with name of organism, stain, date, and student's initials.
2. Gram stain of *Escherichia coli*.
Proceed exactly as with *Bacillus subtilis*.
 3. Gram stain of *Staphylococcus aureus*.
Proceed as with *Bacillus subtilis*.
 4. Gram stain of *Streptococcus lactis*.
Proceed as with *Bacillus subtilis*.

Note: If desired a stain may also be made of a mixture of *Escherichia coli* and *Bacillus subtilis* on another slide, but this should not be done until after the student has become familiar with the appearance of the individual organisms.

C. Examination and Record:

1. Examine with the oil immersion lens as directed in Exercise 11.
2. Record whether organisms are Gram positive or Gram negative.

Note: Organisms retaining the gentian violet stain are spoken of as Gram positive.

3. How is anilin oil gentian violet prepared?
4. What is the composition of Lugol's solution?
5. Is the Gram stain of any practical value? How?
6. What would you suggest as a possible explanation of the reaction of different organisms to the Gram stain?

7. Indicate some other method than that described above for differentiation of Gram positive and negative bacteria.
8. Record the Gram stain for the organism causing the following diseases: pneumonia, gonorrhoea, typhoid, anthrax, tuberculosis, meningitis, cholera, and tetanus.

Ref.: Buchanan. *Bacteriology*, pp. 154, 380, 383-384, 386, 408, 417, 432, 433-434, 440-441.

EXERCISE 13

SPORE STAIN

This exercise illustrates a fundamental characteristic of spores and special methods for their detection. Students should consult the appendix and text for other methods of demonstrating spores.

A. Materials Needed:

1. Agar slant cultures of *Escherichia coli* and *Bacillus subtilis* two to four days old.
2. Carbol fuchsin stain.
3. Five per cent acetic acid.
4. Loeffler's methylene blue stain.
5. Malachite green (5% aqueous solution).
6. Safranin (0.5% aqueous solution).

B. Procedure:

1. To show the presence of spores.
 - a. *Escherichia coli*.
 - (1) Prepare a smear as directed in Exercise 11.
 - (2) Stain with Loeffler's methylene blue for 15 seconds.
 - (3) Wash in water, dry in air or well over the flame.
 - (4) Label with name of organism, type of stain, etc.
 - b. *Bacillus subtilis*.

Proceed as described in "a" above for *Escherichia coli*.
2. To stain the spores.
 - a. Spore stain of *Escherichia coli*.
 - (1) Prepare a smear as directed in Exercise 11.
 - (2) Flood the slide with carbol fuchsin.

- (3) Stain at room temperature over night or heat over the flame until the stain steams and continue to add fresh stain, taking care that no stain is allowed to dry on the slide. Continue heating for two to five minutes.
 - (4) Wash in water.
 - (5) Flood the smear with 5% acetic acid for not more than two seconds.
 - (6) Wash in water.
 - (7) Stain with methylene blue 15 to 20 seconds.
 - (8) Wash in water. Dry in air or well over the flame.
- The following alternative method of staining may be instructive.

- (1) Flood the dried smear with malachite green and heat to steaming three or four times within one-half minute.
- (2) Wash off excess stain for about one-half minute.
- (3) Apply safranin solution for one-half minute.
- (4) Wash in water and dry.

b. Spore stain of *Bacillus subtilis*.

Proceed as with *Escherichia coli* above.

C. Examination and Record:

1. Examine slides with oil immersion lens as in Exercise 11.
2. Make sketch of *Bacillus subtilis* showing spores and vegetative cells and describe using terms below.
 - a. Sporangia (bacteria containing spores):
Medium. age. days.
Form: Elliptical, short rods, spindles, clavate, drumstick.
 - b. Spores:
Form: Spherical, elliptical, elongated.
Location of spores: Central, subterminal, polar.
Diameter: Greater than, less than, or equal to, diameter of vegetative cell.

3. What characteristic of spores is illustrated in their staining properties?
4. How do bacilli, spirilli, and cocci compare as to frequency of spore production?
5. Describe another method for staining spores.

Ref.: Buchanan. *Bacteriology*, pp. 32-35, 153.

Schaeffer and Fulton. 1933. *Science*, **77**, 194.

EXERCISE 14

STAINING METACHROMATIC GRANULES

(Detection of Diphtheria Bacilli)

Special staining methods for demonstration of specific morphological characteristics are performed in this exercise.

A. Materials Needed:

1. Young culture of *Corynebacterium diphtheriae* on Loeffler's blood serum (12 to 18 hours).
2. Loeffler's methylene blue.
3. Albert's stain (solution No. 1 and solution No. 2)

B. Procedure:

1. Loeffler's stain.

- a. Prepare a smear, dry and fix in the flame as directed in Exercise 11.
- b. Flood with Loeffler's methylene blue; allow the stain to act for 3 minutes.
- c. Wash in water.
- d. Dry in the air or well over the flame and label appropriately.

2. Albert's stain.

- a. Prepare a smear, dry and fix as previously directed.
- b. Stain with Albert's solution No. 1 for one minute.
- c. Wash in water.
- d. Dry by blotting with absorbent filter or blotting paper.
- e. Stain with Albert's solution No. 2 for one minute.
- f. Wash and dry with filter paper or blotter and label appropriately.

C. Examination and Record :

1. Examine with the oil immersion lens making a sketch showing the characteristic appearance of the organisms and the metachromatic granules.
2. Are these stains of any practical significance?
3. What is Loeffler's blood serum and how is it prepared?
4. Describe some other method of staining for detection of diphtheria bacilli.

Note : Either of the staining methods given above may be employed for this exercise.

Ref.: Buchanan. *Bacteriology*, pp. 389-392.

EXERCISE 15

CAPSULE STAIN

In this exercise is introduced a different method of fixation than was previously employed.

A. Materials Needed:

1. Twenty-four hour cultures of *Achromobacter viscosum* in milk.
2. Glacial acetic acid.
3. Carbol fuchsin.
4. Physiological salt solution.
5. Coverslips.

B. Procedure:

1. With a platinum loop place five or six loops of the milk culture on the microscope slide.
2. Cover with glacial acetic acid; allow this to act for not more than 10 seconds.
3. Wash off the acid by dropping on carbol fuchsin and allowing the stain to run off.
4. Wash off the carbol fuchsin with physiological salt solution; do not use water.

C. Examination and Record:

1. Place a thin coverslip on the wet stained mount and examine with the oil immersion lens.
2. Sketch, showing bacteria and capsules.
3. What is the function of glacial acetic acid?
4. Is capsule formation of any practical significance? Discuss.
5. Describe briefly another method of staining capsules.

Ref.: Buchanan. *Bacteriology*, p. 27.

EXERCISE 16

THE ACID FAST STAIN

(Test for tubercle bacilli, *Mycobacterium tuberculosis*)

It is the purpose of this exercise to introduce another special staining method and to acquaint the student with the purpose and practice of counterstaining.

A. Materials Needed:

1. Agar cultures of *Mycobacterium smegmatis* and *Staph. aureus*.
2. Sputum from tubercular patient.
3. Staining solution:
 - a. Carbol fuchsin.
 - b. Gabbett's methylene blue.
 - c. Acid alcohol.
 - d. Loeffler's methylene blue.

B. Procedure:

1. Gabbett's method.

- a. Make smear of *Mycobacterium smegmatis*, dry and fix as directed for Exercise 11.
- b. Stain with hot carbol fuchsin for 4 or 5 minutes as for the spore stain.
- c. Wash in water.
- d. Flood the smear with Gabbett's methylene blue; allow to act for 30 seconds.
- e. Wash in water; dry in air or well over the flame.

2. Ziehl Nielsen method.

- a. Stain with carbol fuchsin as directed above.
- b. Decolorize with acid alcohol.
- c. Wash in water.
- d. Stain with methylene blue 15 to 30 seconds.
- e. Wash, dry, and examine.

3. Make stained mount of *Staph. aureus* or a mixture of *Staph. aureus* and *Myco. smegmatis* by either Gabbett's or Ziehl Nielsen's method.
4. Make stained mounts of tubercular sputum by either Gabbett's or the Ziehl Nielsen method.

C. Examination and Record:

1. Examine the slides of *Mycobacterium smegmatis*, using the oil immersion lens.
 - a. What color do the organisms stain?
 - b. Make a sketch showing arrangement and general appearance of the organisms.
2. Examine the other slides as directed for *Mycobacterium smegmatis*.
3. Why are the smegmatis and tuberculosis organisms said to be acid fast?
4. Name two pathogenic acid fast bacteria.
5. Where might you find non-pathogenic acid fast bacteria?

Ref.: Buchanan. *Bacteriology*, pp. 424, 429-431.

EXERCISE 17

MEASUREMENT OF MICROORGANISMS

A. Materials Needed:

1. Stage micrometer.
2. Ocular micrometer.
3. Stained mount of *Bacillus subtilis* or other bacterium.
4. Stained mount of yeast.

B. Procedure and Record:

1. Standardization of ocular micrometer.

- a. With the ocular micrometer in the eye piece and the stage micrometer on the stage, focus with the low power and note the number of spaces of the stage micrometer covered by five or ten spaces of the ocular.
- b. Record the above observation, also the objective lens employed, length of draw tube, number of eye piece, and number of the microscope. Calculate the value of the ocular space in terms of microns.
- c. Repeat the standardization using the high power dry and the oil immersion lens. Record.
- d. Repeat the standardization with the low power, but with the draw tube at 180. Record.
- e. Record.

	OBJECTIVE		
	Low	High Dry	Oil Immersion
Length of draw tube			
Stage micrometer spaces covered by 5 (or 10) ocular spaces			
Value of 1 ocular space in microns			

2. Measurement of microorganisms.

- a. Replace the stage micrometer with a stained mount of yeast or bacteria.
- b. Focus with the oil immersion or high dry lens and record the length and breadth in terms of ocular spaces.
- c. Calculate dimensions in terms of microns.

Note: Approximate magnifications and diameters of fields with $10\times$ ocular and tube length 160 mm. are tabulated below.

OBJECTIVES	MAGNIFICATION (DIAMETERS)	DIAMETER OF FIELD
Low 16 mm. (2/3 in.)	100	2.10 mm.
High dry 4 mm. (1/6 in.)	440	0.40 mm.
Oil immersion 1.8 mm. (1/12 in.)	950	0.20 mm.

C. Report:

1. What was the length and breadth of the cells in ocular spaces and in microns?
2. Define the term micron.
3. What is the effect of extending the draw tube on magnification?
4. How would you proceed to standardize the ocular so as to have one ocular space equal some predetermined length?

EXERCISE 18

MOTILITY OF BACTERIA

(Preparation of the Hanging Drop)

The primary purpose of this exercise is to give practice in preparation of the hanging drop, a technique frequently utilized for diagnosis of disease, as e.g., in the microscopic agglutination test for typhoid fever.

A. Materials Needed:

1. Hollow ground slides (or slides with glass or rubber ring about 1.0 mm. high).
2. Clean cover glasses.
3. Young (not more than 24 hour) broth culture of *Bacillus subtilis*, *Ps. fluorescens*, or *Proteus vulgaris*.
4. Vaseline.

B. Procedure:

1. Make a square or circle with vaseline on the slide around the concave depression as a center (or apply vaseline to the surface of the ring if such slide is used). A film of vaseline about one-sixteenth inch in height is desirable.
2. By means of a small platinum loop place a small drop of a young broth culture of one of the organisms listed, in the center of a clean coverslip which is placed near the edge of the table.
3. Take the hollow ground slide which has previously been prepared as above, pass it quickly through the flame and place it on the coverslip so that the drop of culture will be in the center of the hollow depression and the vaseline will serve as a seal.
4. Turn the slide over. You will now have a drop of the young broth culture suspended in the hollow of the

slide and protected against evaporation by the vaseline seal. Care must be taken that the drop does not touch the slide at any point.

C. Examination and Record:

1. Place the slide on the microscope stage, coverslip uppermost.
2. Focus with the low power of the microscope (16 mm. objective) on the edge of the drop. When this is in focus swing in the high power dry lens (4 mm. objective).

Caution: Do not use the oil immersion lens for this examination. Special care must also be taken as to the intensity of light which is employed. A too intense light is particularly to be avoided.

3. Observe motility and make sketch indicating by a dotted line the path the organism takes in going from one point to another.
4. Define or explain and illustrate the terms (a) flagella, (b) monotrichous, (c) peritrichous, (d) lophotrichous, and (e) amphitrichous.

Ref.: Buchanan. *Bacteriology*, pp. 29-30.

EXERCISE 19

MOTILITY OF BACTERIA IN SOLID MEDIA

(Macroscopic Observation Motility)

A. Materials Needed:

1. Broth culture of *Escherichia coli*.
2. " " " *Bacillus subtilis*.
3. " " " *Staphylococcus aureus*.
4. " " " *Aerobacter (Bact.) aerogenes*.
5. Five tubes nutrient agar (0.5% agar).¹

B. Procedure:

1. Inoculate each organism listed into a separate tube of agar. The inoculation should be made with a straight needle by stabbing, as is directed for gelatin (Ex. 30). Try to make a clean straight stab in the center of the tube about an inch long. (The fifth tube is to serve as a check.)
2. Incubate at the body temperature for 24 hours.

C. Examination and Record:

1. Examine the tubes and record motility. (This is indicated by a clouding of the medium due to the migration of the organism from the line of inoculation.)
2. Illustrate result obtained by sketches.
3. Would you consider this method for determining motility as satisfactory as the hanging drop?

¹ The agar-agar is prepared as in Exercise 3 except that 0.5% agar is used. Tubes should be about one-third full. As soon as the agar is taken out of the autoclave it should be placed in the 37° incubator and left there until the experiment is completed except for the brief period while the tube is being inoculated. It is best to prepare the medium the same day it is to be used.

EXERCISE 20

MOTILITY IN SEWAGE AND HAY INFUSION

A. Materials Needed:

1. Sewage about two days old.
2. Hay infusion three to six days old.
3. Coverslips and slides.

B. Procedure:

1. Make a hanging drop preparation of sewage and hay infusion or put a drop of the liquid on a clean microscope slide and cover with a clean coverslip.

C. Examination and Record:

1. Examine the slide of sewage with the low power, then with the high power dry lens and draw the types of motile organisms seen, as in Exercise 18.
2. Examine the slide of hay infusion as directed for sewage.
3. Are protozoa present in the hay infusion? How do they differ from bacteria?

Ref.: Buchanan. *Bacteriology*, pp. 15-18.

EXERCISE 21

MICROSCOPIC EXAMINATION OF COMPRESSED YEAST AND YEAST FOAM

A. Materials Needed:

1. Fleischmann's or Corby's compressed yeast.
2. Yeast foam.
3. Lugol's solution.

B. Examination of Compressed Yeast:

1. Mix a small amount of material from a cake of compressed yeast with a few drops of water and Lugol's solution on a slide and drop on a coverslip.
2. Examine with low power (16 mm. objective) and make a sketch showing grouping, form of yeast cells, and starch if present.
3. Examine with the high power dry lens and sketch as in 2 above.

C. Examination of Yeast Foam:

1. Make a suspension of yeast foam on a slide as directed for compressed yeast.
2. Add a drop of Lugol's solution. Drop on a cover glass and examine as directed for compressed yeast.
3. Draw starch grains and yeast cells.
4. How may starch grains be differentiated from yeast cells?
5. Describe briefly the method of preparation of yeast foam and compressed yeast.

Ref.: Buchanan. *Bacteriology*, pp. 262-265.

EXERCISE 22

MORPHOLOGY OF YOUNG ACTIVELY GROWING YEASTS

A. Materials Needed:

1. Young 24 hour culture of *Saccharomyces cerevisiae* (or compressed yeast) in beer wort, or glucose yeast water, or preferably malt extract broth. (See Appendix A for preparation.)

B. Procedure:

1. Morphology of *Saccharomyces cerevisiae*:
 - a. By means of a platinum loop or pipette place a little of the sediment of the culture furnished on a slide. Cover with a clean coverslip.

C. Examination and Record:

1. Examine with the low power. Sketch.
2. Examine with the high power dry lens. Sketch. Note the method of multiplication, presence of granules, vacuoles, nuclei, etc., and indicate in your sketch.
3. Remove the coverslip. Allow the material remaining on the slide to dry. Fix, and stain with dilute carbol fuchsin (10%) for two seconds.
4. Examine this stained mount with the oil immersion lens. Draw. Note size as compared with bacteria.
5. How do yeasts multiply? Is there any evidence of this in the culture studied?
6. Differentiate yeasts from bacteria.
7. What is meant by the term torula?

Ref.: Buchanan. *Bacteriology*, pp. 60-71.

EXERCISE 23

DISTRIBUTION AND ISOLATION OF YEASTS

This exercise is intended to demonstrate the ubiquity of yeasts and the possible use of the petri plate as a means of determining the relative incidence of microorganisms in the air.

A. Materials Needed:

1. Four tubes of malt extract or beer wort agar.¹
2. Four tubes of beer wort or malt extract broth.¹
3. Ripe grapes, apples, banana, unbleached raisins, etc.
4. Four sterile petri dishes.

B. Procedure:

1. Isolation of yeasts from the air.

- a. Melt the tubes of agar medium and pour each into separate petri dishes. Expose the dishes (open) in different parts of the room or elsewhere for 15 minutes.
- b. Place in the locker until the next period.

2. Distribution of yeasts in nature.

- a. Inoculate one tube of liquid medium with a little soil.
- b. To another tube of liquid medium add some ripe grapes, if available.
- c. To the remaining tubes of liquid media add a few unbleached raisins, bits of apple with skin, or other fruit.
- d. Keep in the locker until the next period.

¹ Difco dehydrated malt extract agar and broth have been found very satisfactory.

C. Examination and Record:**1. Examination of plates exposed to air.**

- a. Record (in tabular form) the number of yeasts, molds, and other colonies appearing on each plate.
- b. Make a stained mount of several colonies that appear to be yeasts in order to determine whether yeasts are present.

2. Examination of tubes of beer wort (or malt extract broth).

- a. Smell each tube to see if it has an odor of alcohol.
 - b. Place a drop of the sediment on a slide, cover with a thin coverslip and examine with the low and high power dry lenses. Record if yeasts are present and sketch.
 - c. Make a stained mount of the sediment. Examine with the oil immersion lens and record if yeasts are present.
 - d. Test for the presence of alcohol by the iodoform reaction as follows: add NaOH, then iodine crystals, heat and note if there is present the characteristic odor of iodoform.
- 3. What is meant by spontaneous alcoholic fermentation? What groups of organisms are concerned and where do they come from?**
- 4. Calculate the number of yeasts and other cells that were falling per square foot per hour in the various petri dishes exposed. Record in tabular form.**

EXERCISE 24

STUDY OF YEAST SPORES

(Plaster Paris Blocks Method)

A. Materials Needed:

1. Young vigorously growing culture of *Saccharomyces cerevisiae*. This is obtained by transferring to beer wort (or malt extract broth) every day for three or four days.
2. Sterile petri dishes containing blocks of sterile plaster paris.
3. Sterile distilled water two tubes (10 c.c.).
4. Sterile pipettes.

B. Procedure:

1. Pour a tube of distilled water into each of two petri dishes containing the sterile plaster paris blocks. (Observe all precautions of sterility; the plug should be flamed and the mouth of the tube thoroughly sterilized.)
2. By means of a sterile pipette transfer a small amount of sediment of the pure cultures to plaster paris blocks. This may be done in the following manner: Take the pipette between the thumb and middle finger of the right hand, place the forefinger tightly over the upper opening and insert the pipette into the tube of beer wort so that the delivery end touches the bottom of the tube. Now raise the forefinger and the sediment will rise into the pipette. Replace the forefinger, withdraw the pipette, and inoculate the plaster paris blocks.
3. Place in the locker for 48 to 72 hours. (Do not store in the 37° incubator.)

C. Examination and Record:

1. Make a smear from the material on the plaster paris block as directed for making bacterial mounts in Exercise 11.

2. Dry and fix.
3. Stain with hot carbol fuchsin for five minutes.
4. Wash in water.
5. Decolorize with 5% acetic acid for a second or two.
6. Wash in water.
7. Stain with methylene blue for 15 seconds.
8. Wash in water.
9. Dry.
10. Examine with the oil immersion lens.
11. Sketch, showing spores, their arrangement, number in cell, etc.
12. Define ascospore and compare spore formation in yeast with that in bacteria.

Ref.: Buchanan. *Bacteriology*, pp. 62-64.

EXERCISE 25

YEAST SPORE FORMATION ON CARROT AGAR

A. Materials Needed:

1. Culture of yeast or yeast cake.
2. Carrot agar slant.¹ (One for each culture employed.)

B. Procedure:

Inoculate each of the yeast cultures furnished onto carrot agar and incubate in locker for 2 to 14 days.

C. Examination and Record:

1. After two or three days, and again after a week or two, make a spore stain as directed in the preceding exercise.
2. Examine with the oil immersion lens, record presence or absence of spores, and sketch.

¹ See Appendix for preparation.

EXERCISE 26

MORPHOLOGY OF MOLDS

It is the function of this experiment to familiarize the student with the principal characteristics to be looked for in describing the common molds.

A. Materials Needed:

1. Plate cultures of *Aspergillus* or *Penicillium*.
2. Plate cultures of *Mucor* or *Rhizopus*.
3. Slides and coverslips.

B. Examination and Record:

The following outline is to be followed in studying and describing molds in this and succeeding exercises.

For this exercise two molds are to be studied, *Aspergillus* or *Penicillium*, and *Mucor* or *Rhizopus*.

1. Gross structure on plate.

Examine plates of molds with the naked eye and record:

- a. *Size*: Approximate area of single colony in sq. cm.
- b. *Character of growth*: Smooth, cottony, velvety, dusty, etc.

2. Microscopic examination.

- a. Remove the cover from the petri dish and examine molds on the plate with the low power (16 mm.).

A binocular dissecting microscope will be found very useful for demonstration of natural growth characteristics. (Petri dishes should not be open any longer than is absolutely necessary.)

- b. Remove a small portion of the mold (select the younger part of the colony) to a drop of alcohol on a slide, then add a drop of water, and cover with a thin coverslip.

EXERCISE 27

STUDY OF MOLDS FROM AIR

It is the purpose of this and the two following exercises to acquaint the student with the use of a dichotomous key for identification of mold genera and to demonstrate the ubiquity of these organisms.

A. Materials Needed:

1. Four tubes of beer wort agar (or malt extract agar).
2. Four sterile petri dishes.
3. Slides and coverslips.

B. Procedure:

1. Melt the tubes of agar and pour each into a separate petri dish.
2. Expose dishes to infection as follows:
 - a. Leave one open some place outside the laboratory for 15 minutes.
 - b. Place one out in the street or on the sill of an open window.
 - c. Shake a rag over another.
 - d. Cough or shake hair into the fourth.
3. Place in the 37° incubator until the next period (preferably 2 to 4 days).

C. Examination and Record:

1. Count the number of molds that have developed on each petri dish, and tabulate.
2. Calculate the number of mold spores that fall per square foot per hour in each of the places of exposure; tabulate.
3. From the naked eye examination indicate the number of apparently different kinds of molds that developed.

4. Select two molds of different appearance and identify them, using as a guide the study made of *Aspergillus* and *Mucor* in Exercise 26 and the appendix in Buchanan's *Bacteriology*. Record their characteristics in tabular form.

EXERCISE 28

STUDY OF MOLDS ON DECAYED FOODS

A. Materials Needed :

1. Orange.
2. Boiled Irish potato.
3. Boiled sweet potato.
4. Prunes.
5. Moist bread.
6. Tomato.
7. Peas.
8. Seven sterile petri dishes.

B. Procedure :

1. Place in each of seven petri dishes pieces of blotting paper or several pieces of filter paper; moisten with tap water.
2. Put a small portion of each of the above foods in separate petri dishes.
3. Set in the locker until the next period.

C. Examination and Record :

1. Record when molds have developed and whether the growth of the molds is restricted to the food or if it extends far beyond.
2. Do you notice any gross differences in the types of molds? Record the apparent number of different types of molds that developed on the different materials.
3. Save material for the next exercise.

EXERCISE 29

IDENTIFICATION OF MOLDS FROM FOODS

A. Materials Needed:

1. Molds from preceding exercise.
2. Camembert or Roquefort cheese.
3. Sharp knife or scalpel.

B. Examination and Record:

1. Select several molds growing on decaying food (see previous exercise).
2. Examine as described in Exercise 26 and determine the genus to which they belong with the aid of the key to molds.
3. Examine thin slices of Camembert or Roquefort cheese and identify the genus of the molds present.

Ref.: Buchanan. *Bacteriology*, pp. 501-517; Key to the Families and Genera of Common Molds.

EXERCISE 30

STUDY OF CULTURAL CHARACTERS OF BACTERIA

(Methods of Inoculation and Transfer)

The function of this exercise is twofold—(1) to instill the proper technique for making a transfer of bacterial cultures, and (2) to acquaint the student with the mass appearance of bacterial growths on a few simple culture mediums and the methods for describing such growths.

A. Materials Needed:

1. Young agar slant cultures of the following organisms:

- a. *Escherichia coli*.
- b. *Bacillus subtilis*.
- c. *Streptococcus lactis*.
- d. *Serratia marcescens* (*Erythrobacillus prodigiosus*).

Note: These cultures or transfers made from them from time to time are to be used for a considerable number of subsequent exercises. The student will be held responsible for the possession of these cultures in a pure state. The table should be wet down, preferably with a disinfecting solution, before beginning transfers.

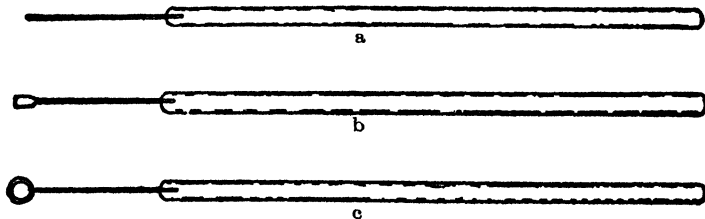


FIG. 2. PLATINUM WIRES AND LOOPS USED IN THE LABORATORY.
a, straight wire; b, spatulate wire; c, platinum loop or oese.

2. Five tubes of plain agar slants.
3. Five tubes of gelatin.
4. Five tubes of potato slants.
5. Five tubes of broth.

B. Procedure:

1. Inoculation.

Note: Methods outlined below for transferring and inoculating cultures should be memorized and practiced until the student becomes proficient in carrying them out. Proper performance of the work in this exercise is fundamental to all subsequent bacteriological work.

a. To inoculate an agar slant:

- (1) Place the agar slant of *Escherichia coli* and the agar slant to be inoculated between the thumb and forefinger of the left hand, the cotton plugs facing toward the body. Bring the thumb and forefinger together and interpose the middle finger between the two tubes. Now close the hand. This will give one tube resting between the fore and middle fingers and the other between the middle and third fingers. At the base both tubes are held firmly by the thumb and forefinger.
- (2) Singe the cotton plugs. This removes any dust that might have accumulated upon them.
- (3) Take the straight platinum needle and sterilize it in the gas flame by heating to red heat. (Also pass the handle of the needle through the flame.) The needle should be held as one does a pencil.
- (4) Remove the cotton plugs by grasping them with the backs of the unoccupied fingers of the right hand.
- (5) Flame the mouths of the tubes.
- (6) With the sterile platinum needle touch lightly the growth in the tube of *Escherichia coli*.

- (7) The needle which now carries upon it some of the culture to be transferred is introduced into the second tube of agar and a streak made upon the surface. Care should be taken not to puncture the medium and inoculation should be made in a single straight line from the base to the top of the slant.
- (8) Flame the mouths of the tubes and replace the cotton plugs.
- (9) Sterilize the platinum needle before laying it down. Care should be taken that the needle does not touch any part of the furniture during the process.
- (10) Label tube with name of organism, date, medium, temperature of incubation, and student's initials.

b. Inoculation of potato slant:

- (1) This is carried out in exactly the same manner as for agar

c. To make a gelatin stab:

- (1) Instead of streaking on the surface, the transferred material is stabbed into the medium to a depth of an inch or more. Otherwise the procedure is identically the same as that for agar slant cultures.

d. Inoculation of broth:

- (1) The broth is inoculated as described for gelatin. The needle should then be shaken in the medium.

Note: The student will make transfers from each of the organisms furnished, in the manner described above.

2. Incubation (48 hours or until next period).

Gelatin liquefies at temperatures above 22° to 25° C. and should therefore be incubated in the 20° ther-

mostat. All cultures of *Serratia marcescens* should also be incubated at 20° or in the locker. All other materials are to be incubated at the body temperature (37° C.).

C. Examination and Record:

1. Agar streak.

Examine each culture, sketch and record in tabular form with the following as a guide. (Terms employed are defined in the Appendix.)

Growth: Scanty, moderate, abundant, none.

Form of growth: Filiform, echinulate, beaded, spreading, arborescent, rhizoid, plumose.

Elevation of growth: Flat, effuse, raised, convex.

Luster: Glistening, dull, cretaceous.

Topography: Smooth, contoured, rugose, verrucose.

Optical characters: Opaque, translucent, opalescent, iridescent.

Chromogenesis: Red, yellow, green, brown, fluorescent, etc.

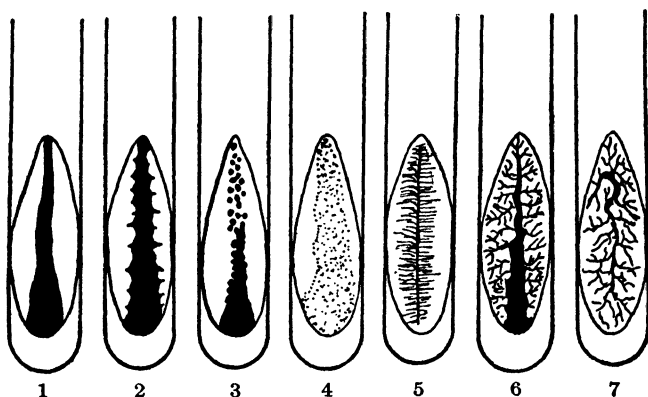


FIG. 3. FORMS OF GROWTH ON STREAK CULTURES.

1, filiform; 2, echinulate; 3, beaded; 4, effuse; 5, plumose;
6, arborescent; 7, rhizoid.

Odor: Absent, decided, resembling

Consistency: Slimy, butyrous, viscid, membranous, brittle.

Medium: Grayed, browned, reddened, blued, greened, unchanged.

2. Potato.

Sketch and record each culture as directed for agar.

3. Nutrient broth.

Sketch and record as below:

Surface growth: Ring, pellicle, flocculent, membranous, none.

Clouding: Slight, moderate, strong, transient, none.

Odor: Absent, decided, resembling

Sediment: Compact, flocculent, granular, flaky, viscid on agitation, abundant, scant.

4. Gelatin stab.

Sketch and record as below:

Growth: Uniform, best at top, best at bottom.

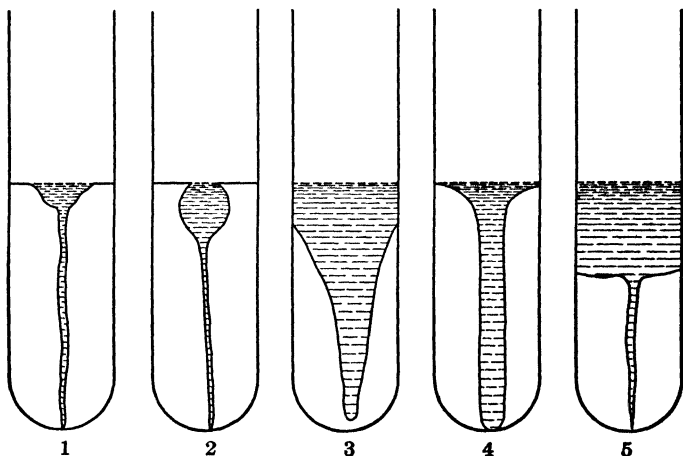


FIG. 4. TYPES OF LIQUEFACTION IN GELATIN STAB CULTURES.

1, crateriform; 2, napiform; 3, infundibuliform; 4, saccate; 5, stratiform.

CULTURAL CHARACTERS OF BACTERIA 73

Line of puncture: Filiform, beaded, papillate, villous, plumose, arborescent.

Liquefaction: Crateriform, napiform, infundibuliform, saccate, stratiform, none, slow, rapid.

Medium: Fluorescent, browned, unchanged.

Ref.: Buchanan. *Bacteriology*, pp. 127-138.

EXERCISE 31

STUDY OF CULTURAL CHARACTERS OF YEAST

A. Materials Needed:

1. Cultures of *Sac. cerevisiae* (or *Sac. ellipsoideus*) and *Torula rosea*.
2. Three tubes of wort, malt extract broth, or yeast water.
3. Three tubes of dextrose agar slants.
4. Three tubes of plain agar slants.
5. Three tubes of beer wort or malt extract agar slants.
6. Three tubes of nutrient gelatin.

B. Procedure:

1. Inoculate one tube of each material with the culture of *Sac. cerevisiae* (or *Sac. ellipsoideus*) as described for transferring bacteria in Exercise 30.
2. Inoculate a set with *Torula rosea* as above.
3. Incubate at room temperature (in locker) for 48 hours or until the next period. (It is best to incubate gelatin in the 20° C. thermostat.)

C. Examination and Record:

1. Examine and record as outlined for bacterial cultures in Exercise 30.
2. Compare vigor of growth on the different media.

EXERCISE 32

ANAEROBIC CULTURE METHODS

The object of this exercise is to introduce one of the more common methods for producing anaerobic conditions.

A. Materials Needed:

1. Young cultures of *Escherichia coli*, *Serratia marcescens*, *Bacillus subtilis*, and *Clostridium sporogenes*.
2. Eight agar slants (must be free from glucose or other sugars).
3. Four per cent NaOH solution.
4. Pyrogallic acid.
5. Cork stoppers.

B. Procedure:

1. Effect of air supply on growth of *Bacillus subtilis*.
 - a. Inoculate *Bacillus subtilis* onto two agar slants.
 - b. Push the cotton plug of one of these slants into the neck of the tube until it nearly reaches the agar, then fill the tube with pyrogallic acid. This may readily be done by plunging the tube (mouth down) into a can of pyrogallic acid.
 - c. Select a cork stopper which fits smoothly into the mouth of the tube.
 - d. Add about 2 c.c. of 4% NaOH.
 - e. Quickly cork the tube and invert.
Incubate at 37° C. together with the other tube of *Bacillus subtilis* until the next period. (Preferably 24 hours and not more than 48 hours.)
2. Effect of air supply on growth of *Escherichia coli*.
Proceed with *Escherichia coli* as directed for *Bacillus subtilis*.
3. Effect of air supply on growth of *Cl. sporogenes*.
Proceed as directed for *Bacillus subtilis*.

4. Effect of air supply on growth and pigment production of *Serratia marcescens*.

Proceed as directed for *Bacillus subtilis*, but incubate in the locker.

C. Examination and Record:

1. Examine the anaerobic and aerobic tubes of *Bacillus subtilis* and record in which growth is most abundant.
2. Repeat with *Escherichia coli* and *Cl. sporogenes* as directed for *Bacillus subtilis*.
3. Repeat with *Serratia marcescens* and record also in which tube pigment formation is most intense.
4. What is the effect of air supply on pigment production of *Serratia marcescens*?
5. Define the terms aerobe, anaerobe, facultative, and microaerophile.
6. Illustrate by chemical reactions (a) an anaerobic and (b) an aerobic bacterial decomposition.
7. Describe some other method for making anaerobic cultures.

Ref.: Buchanan. *Bacteriology*, pp. 176-178, 269-280.

EXERCISE 33

ISOLATION OF PURE CULTURES AND STUDY OF COLONY CHARACTERISTICS OF *ESCH.* *COLI* AND *B. SUBTILIS* ON AGAR PLATES

The technique here employed is applicable to the isolation of an organism from a mixture in which it is present in excess. The fact that bacteria may produce very distinctive mass growths (colonies) will also be evident.

A. Materials Needed:

1. Twenty-four to 48 hour old broth culture of *B. subtilis*.
2. Twenty-four to 48 hour old broth culture of *Esch. coli*.
3. At least eight clean, sterile petri dishes.
4. Eight tubes three-fourths full of agar.

Note: A plate culture of an organism is one so prepared that the individual bacteria used for inoculation are separated from each other by appreciable distances. This is accomplished by mixing the bacteria with liquid nutrient gelatin or agar and pouring the medium so that it will spread over a considerable horizontal surface. The medium cools and solidifies. The bacteria are thus more or less uniformly separated from each other. They begin to grow and their progeny form masses large enough to be seen with the naked eye. Such masses are called "colonies." The character of these colonies differs with different species of bacteria. The plate culture method is used for at least three distinct purposes:

- (1) To allow for the formation of typical colonies which may be studied.

- (2) To enable one to separate different kinds of bacteria when mixed together and thus secure pure cultures.
- (3) To determine the number of bacteria present in any substance. (See Ex. 68.)

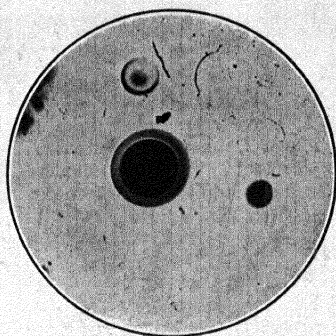
B. Procedure:

1. Sterilize your petri dishes for one and one-half to three hours at 150° C. to 160° C. in the hot air oven if not sterilized at the last exercise, and label them from one to eight.¹
2. Label the tubes of agar from one to eight and place them in a water bath; boil until melted; cool to 45° C.
3. Inoculate tube No. 1 with a loopful of the medium from a broth culture of *B. subtilis*. Roll tube No. 1 between the palms of the hands to distribute the bacteria uniformly.
4. Inoculate tube No. 2 with three loopfuls of the medium from tube No. 1, mix thoroughly and replace tube No. 2 in the water (at 45° C.).
5. Pour tube No. 1 into petri dish No. 1 as follows:

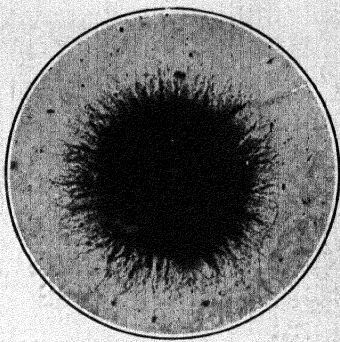
Take the tube of agar in the right hand, remove the cotton plug and thoroughly flame the mouth of the tube. When the glass is again cool pour the contents of the tube into the lower half of the petri dish, raising the upper half no more than is necessary to insert the tube. Tilt the dish back and forth carefully until the entire bottom of the dish is covered with the agar, taking care not to have any agar run over the edges of the dish.

6. Transfer three loops of media from tube No. 2 to tube No. 3, and pour tubes No. 2 and No. 3 into petri dishes No. 2 and No. 3 respectively as directed above.
7. Pour tube No. 4 into petri dish No. 4. This serves as a control and constitutes a check on your care and technique.

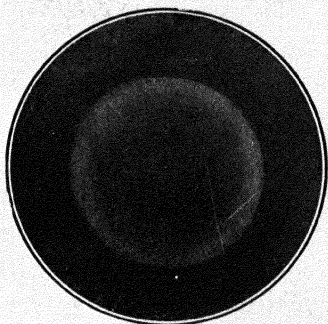
¹ The table should be wet down (preferably with a disinfecting solution) before beginning this exercise and windows must be closed.



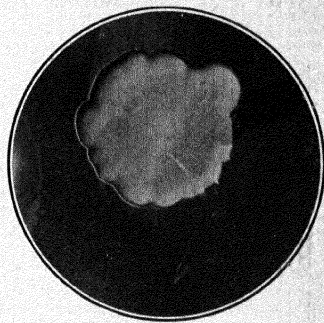
1



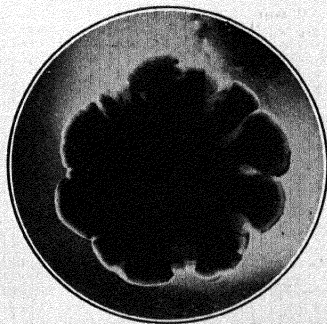
2



3



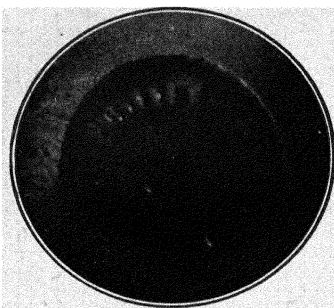
4



5

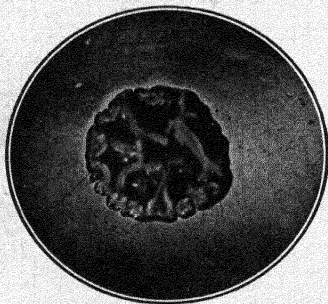
1, circular with entire margin;
2, myceloid; 3, circular; 4, circular
with lobate margin; 5, circular with
auriculate margin.

FIG. 5. TYPES OF BACTERIAL COLONIES.



1

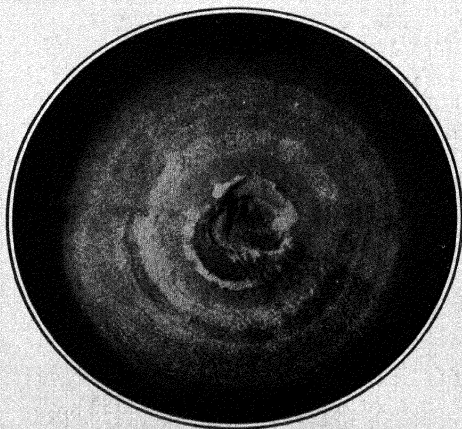
1, circular, radial wrinkling of surface; 2, thick, irregular wrinkling of surface; 3, wrinkling not extending to margin; 4, concentrically ringed colony.



2



3



4

FIG. 6. TYPES OF BACTERIAL COLONIES.

8. Pour plates with *Esch. coli* as directed for *B. subtilis* above, labeling tubes and petri dishes, 5, 6, 7, and 8.¹
9. After the agar has solidified, invert the petri dishes and incubate at 37° C.

Note: If a broth culture is not at hand, a suspension of an agar slant culture in sterile water or physiological salt solution may be used.

The inoculation of the tubes and pouring of the plates must be done very quickly, for if the temperature of the agar falls below 40° C. the agar may solidify. Keep the agar plates in the warm incubator (37° C.) until the next period, if not more than 48 hours; otherwise remove them to the ice box after 24 hours and examine them at the next period.

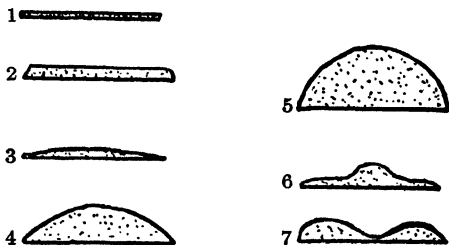


FIG. 7. CROSS SECTION OF VARIOUS TYPES OF COLONIES.

1, flat; 2, raised; 3, convex; 4, pulvinate; 5, capitate; 6, umbonate; 7, umbilicate.

C. Examination and Record:

1. Count and record the number of colonies on each plate.
2. Draw and describe a surface and sub-surface colony of each culture as follows:

Size: Estimate diameter in mm.

Form: Punctiform, circular, irregular, myceloid, filamentous, rhizoid.

¹ If apparatus is available, another series of plates including a mixture of *Esch. coli* and *B. subtilis* will be found very instructive, or a mixture of the two cultures may be substituted for either.

Surface: Smooth, rough, concentrically ringed, radiate.

Elevation: Flat, effuse, raised, convex, umbonate.

Edge: Entire, undulate, lobate, erose, filamentous, curled.

Internal structure: Amorphous, finely or coarsely granular, filamentous, curled, concentric.

Ref.: Buchanan. *Bacteriology*, pp. 122-126.

EXERCISE 34

ACID AND GAS PRODUCTION FROM GLUCOSE IN LIQUID MEDIA

It is the purpose of this experiment (1) to acquaint the student with the use of the Smith fermentation tube and the Frost gasometer, and (2) to illustrate the action of some bacteria on a carbohydrate.

A. Materials Needed:

1. Young culture of *Esch. coli*.
2. Young culture of *B. subtilis*.
3. Four tubes (dextrose) glucose broth or glucose peptone water in Smith tubes.
4. Frost gasometer.

B. Procedure:

1. Inoculate two tubes of freshly prepared broth with *Esch. coli* and two with *B. subtilis*. Leave in 37° incubator for 24 to 48 hours.

C. Examination and Record:

1. Measure the amount of gas formed with a Frost gasometer and record.
2. Add a few drops of litmus or preferably one-half c.c. of brom cresol purple or brom thymol blue to each and record whether the medium is acid or alkaline.
3. If gas is formed determine the composition as follows:
Remove the cotton plug and fill the open arm of the gas tube with 4% NaOH.
 - a. Place the thumb over the open end and manipulate the tube so that the gas will pass from the closed into the open arm.

- b. Without removing your thumb manipulate the tube so that the gas goes back into the closed arm. Now remove the thumb.
- c. Any CO_2 which was present will have been absorbed, thus creating a vacuum and the liquid will be seen to rise in the closed tube after the thumb is removed.
- d. Remeasure the gas, and record.

The residual gas is for the most part hydrogen, which should be tested for by bringing it into the open arm and placing the mouth of the tube in the gas flame. The characteristic explosion of the hydrogen-oxygen mixture will be noted.

4. Tabulate the results under the following headings:
 - a. Growth in the open arm of the Smith tube.
 - b. Growth in the closed arm of the Smith tube.
 - c. Reaction.

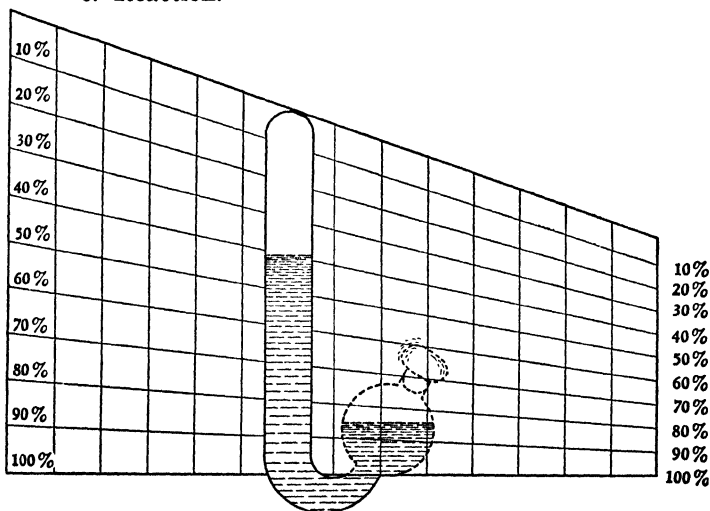


FIG. 8. GASOMETER (FROST'S). THE AMOUNT OF GAS PRODUCED IN THE FERMENTATION TUBE IS READ OFF DIRECTLY FROM THE GASOMETER.

- d. Gas production.
- (1) Per cent total.
 - (2) Per cent hydrogen.
 - (3) Per cent carbon dioxide.
 - (4) Ratio of H:CO₂.
5. Is the Smith tube applicable for the study of aerobiosis and anaerobiosis? Give reasons for your answer.

Ref.: Buchanan. *Bacteriology*, pp. 139-144.

EXERCISE 35

ACID AND GAS FORMATION IN SOLID MEDIA

(Lactose Agar)

The technique employed in this exercise introduces what is known as the "shake culture." The differentiation of bacteria on the basis of their ability to decompose a carbohydrate and the products formed are also illustrated.

A. Materials Needed:

1. Young cultures of *Esch. coli*, *B. subtilis*, and *Strep. lactis* on agar or in milk.
2. Four tubes of lactose agar containing Andrade indicator.
3. Four tubes of lactose agar (10 c.c.).
4. One tube of litmus.
5. Sterile pipettes in a case.

B. Procedure:

1. Melt the lactose agar, cool to 65° C. and to each of the four tubes (containing no indicator) add 1 c.c. of litmus.
2. Cool to 42° C. and inoculate one tube of litmus and one of Andrade lactose agar with *Esch. coli*.
3. Inoculate *B. subtilis* and *Strep. lactis* as was done with *Esch. coli*. The remaining tubes are controls.
4. Incubate at 37° C. 24 to 48 hours or until the next period.

Note: Inoculation must be made quickly while the medium is still liquid, but the temperature should not be above 42° C. Immediately after inoculation the tube should be rolled between the palms of the hands to distribute the organisms.

C. Examination and Record :

1. Record in tabular form:
 - a. Gas production.
 - b. Acid or alkali production.
 - c. Decolorization of the indicator.
 - d. Whether growth is best at top, bottom, or evenly distributed.
2. Which indicator do you consider preferable for this experiment? Give reasons.
3. How is the Andrade indicator prepared?
4. Can the "shake culture" be employed to ascertain whether an organism is aerobic or anaerobic? Explain.

Ref.: Buchanan. *Bacteriology*, pp. 142, 269, 277-278.

EXERCISE 36

GAS FORMATION BY YEASTS

This exercise illustrates an important physiological activity of yeast, which is of practical use in the home. (CO₂ production.)

A. Materials Needed:

1. Compressed yeast.
2. Culture of *Saccharomyces cerevisiae* or *Saccharomyces ellipsoideus*.
3. Two tubes of beer wort or malt extract broth in Smith tubes, or yeast water with 5% glucose.

B. Procedure:

1. Inoculate heavily one tube of beer wort (or malt extract broth) with a bit of compressed yeast and another with some of the pure culture furnished.
2. Leave in the locker for 48 hours or until the next period. (Do not place in the body temperature incubator.)

C. Examination and Record:

1. Measure the amount of gas formed by use of the Frost gasometer as indicated in Exercise 34.
2. Determine the composition of the gas as described in Exercise 34.
3. Tabulate results.
4. Illustrate by a chemical formula alcohol and gas production from glucose by yeast.
5. In what respect does gas production from glucose by yeast and *Esch. coli* differ?

Ref.: Buchanan. *Bacteriology*, p. 224.

EXERCISE 37

DETECTION OF SPORE FORMERS

A. Materials Needed:

1. Broth culture of *B. subtilis* at least three to seven days old.
2. Broth culture of *Esch. coli* at least three to seven days old.
3. Two tubes of broth.

B. Procedure:

1. Place two tubes of broth in a water bath at 80° C.
2. Add to one tube of broth a loop or two of the culture of *B. subtilis*. (Be careful not to touch the sides of the tube.)
3. Inoculate the other tube of broth with a loop or two of the old culture of *Esch. coli*.
4. Keep the tubes in the water bath at 80° C. for ten minutes, then remove them to a can of tap water in order to cool rapidly.

Note: The height of water in the bath must be well above that of the medium in the tubes.

5. Incubate at the body temperature for 48 hours or until the next period.

C. Examination and Record:

1. Examine the tubes of broth inoculated with *Esch. coli* and *B. subtilis*, respectively, and record whether characteristic growth has occurred. Explain the results.
2. What is the principle involved in the technique for isolation of spore formers?
3. How would you proceed to isolate spore forming bacteria from soil or from milk?

EXERCISE 38

INDOL PRODUCTION BY BACTERIA

In this exercise, the principle of differentiation of bacteria through the detection of a specific metabolic end product, "Indol," and tests for this substance are introduced.

A. Materials Needed:

1. Eight tubes of peptone (Dunham's solution) or preferably tryptophane broth.
2. Cultures of *Esch. coli* and *B. subtilis*.
3. Fresh sewage.
4. H_2SO_4 —10% solution.
5. NaNO_2 —1/10,000 freshly prepared.
6. Paradimethylaminobenzaldehyde solution.
7. Saturated potassium persulphate solution.
8. Kovac's Indol Reagent.
9. Water bath.
10. Absorbent cotton.

B. Procedure:

1. Inoculate two tubes of medium with *Esch. coli*.
2. Inoculate two tubes of medium with *B. subtilis*.
3. Inoculate two tubes of medium with sewage.
4. The remaining tubes are to be employed as controls.
5. Incubate at 37° C. for at least 48 hours, preferably four to five days.

C. Examination and Record:

1. Divide one culture of *Esch. coli* into two portions.
 - a. To one portion add one-half c.c. of 10% H_2SO_4 ; mix; then add carefully one c.c. of 1/10,000 NaNO_2 so as to form a layer on the surface. If indol is present a red ring should form at the juncture of the peptone and NaNO_2 in two to twenty minutes.

- b. To the other portion add 0.2 to 0.4 c.c. of Kovac's reagent: Presence of indol will be indicated by red coloration on the surface.
2. With the other tube of *Esch. coli*, proceed as follows:
 - a. Place a few drops of a saturated solution of potassium persulphate, then a few drops of paradimethylaminobenzaldehyde, on a piece of absorbent cotton.
 - b. With the aid of a glass rod or the end of the handle of a platinum needle insert the cotton into the tube, pushing it down until the moistened portion is within about one inch of the surface of the culture medium.
 - c. Place the tube in a boiling water bath (or preferably in a bath at a temperature just below the boiling point) for 5 to 10 minutes. If indol is present a pink to red color will appear on the bottom of the cotton plug.
3. Repeat the above tests with *B. subtilis*, sewage, and the controls.
4. Tabulate your results.
5. Write the formula for indol and nitroso-indol.
6. What is the source of indol in culture media?
7. Where would you expect to find indol in nature? Why?
8. Discuss the relative specificity of the indol tests used.

Ref.: Buchanan. *Bacteriology*, p. 146.

EXERCISE 39

REDUCTION OF NITRATES TO NITRITES

A. Materials Needed:

1. Culture of *Strep. lactis* or *Micrococcus luteus*.
2. Culture of *B. subtilis* or *B. mycoides*.
3. Culture of *Esch. coli*.
4. Four tubes of nitrate broth solution.

If desired, fermentation tubes may be employed to detect production of gas (i.e., complete reduction).

5. Sulphanilic acid.
6. Naphthyl-amine-acetate.

B. Procedure:

1. Inoculate a tube of nitrate broth with *B. subtilis*, or *B. mycoides*, another with *Strep. lactis*, or *Micro. luteus*, and a third with *Esch. coli*. The fourth should not be inoculated and is to serve as a check.
2. Incubate at the body temperature for 48 hours or until the next period.

C. Examination and Record:

1. To each tube add 1 c.c. of sulphanilic acid and naphthyl-amine-acetate. The reduction of nitrate to nitrite is indicated by the production of a distinct red coloration. Comparison should be made with the blank as small quantities of nitrites are apt to be present in nitrates as impurities.
2. Write an equation illustrating the reduction of nitrates to nitrites.
3. Is this change more likely to take place (a) under aerobic or anaerobic conditions, (b) in dry or water-logged soil?
4. Discuss the significance of this reaction in nature.

Ref.: Buchanan. *Bacteriology*, pp. 145, 204, 247-248.

EXERCISE 40

PRODUCTION OF HYDROGEN SULPHIDE

A. Materials Needed:

1. Culture of *Esch. coli*.
2. Culture of *Proteus vulgaris* or *Citrobacter sulphidogenes*.
3. Three tubes of proteose peptone iron-citrate agar about one-half full.

B. Procedure:

1. Inoculate one tube with *Esch. coli* by stabbing into the center of the medium and also along the outer periphery of the cylinder of media where it is in contact with the tube. Make deep stab inoculations.
2. Inoculate another tube with *Proteus vulgaris* or *Citrobacter sulphidogenes* as directed above.
3. The third tube serves as a control.
4. Incubate at body temperature for 48 hours.

C. Examination and Record:

1. Record any visible evidence of H_2S production.
2. Explain results.

EXERCISE 41

ACETIC FERMENTATION

In this and the four succeeding exercises, the economic importance of bacteria and some of the more important carbohydrate fermentation products are brought to the attention of the student.

A. Materials Needed:

1. Sterile cider, other fruit juice or beerwort in Erlenmeyer flasks.
2. Cultures of yeast and *Acetobacter aceti* or mother of vinegar.

B. Procedure:

1. Inoculate the flask of cider (or other material furnished) with the yeast and acetic acid bacteria, or with mother of vinegar.
2. Incubate in the locker for several weeks.

C. Examination and Record:

1. Prepare a stained mount from the film on the material inoculated above. Draw showing characteristic forms. (Note if involution forms are present.)
2. Record any evidence of acetic acid.
3. Write the reaction for the oxidation of alcohol to acetic acid.
4. If time permits determine the amount of acetic acid formed by titrating with a known alkali. (Assume that all of the acid is acetic.)
5. Describe briefly a commercial method of vinegar production.

Ref.: Buchanan. *Bacteriology*, pp: 281-288:

EXERCISE 42

OXALIC FERMENTATION

A. Materials Needed:

1. Old beer wort agar plate of *Aspergillus*.

B. Examination and Record:

1. Examine with the low power microscope, searching carefully in the medium, for crystals of calcium oxalate.
2. Draw the crystals of calcium oxalate.
3. Write structural formula for oxalic acid.
4. Write reaction for the transformation of glucose to oxalic acid.

Ref.: Buchanan. *Bacteriology*, pp. 290-291.

EXERCISE 43

LACTIC FERMENTATION AND ACTION OF BACTERIA ON MILK

A. Materials Needed:

1. *Strep. lactis* (in milk).
2. *Esch. coli* (on agar).
3. *Lactobacillus bulgaricus* (in milk).
4. *Achro. viscosum* (in milk).
5. *B. subtilis* (on agar).
6. Six tubes of litmus milk or brom-cresol-purple milk.

Note: Either brom-cresol-purple or litmus milk may be employed in this experiment. Brom-cresol-purple for this exercise is prepared as follows: Grind 0.5 grams of the indicator in a glass or agate mortar. Dissolve it in 14 c.c. of N/10 NaOH and dilute to 100 c.c. with distilled water. Use 1% of this indicator in milk. (10 c.c. per liter.)

B. Procedure:

1. Inoculate each culture enumerated above into separate tubes of litmus or brom-cresol-purple milk.
2. Incubate at body temperature for 24 to 48 hours or until the next period.

C. Examination and Record:

1. Examine the tubes of milk and record (in tabular form) after 24 to 48 hours.
 - a. Consistency.
 - b. Coagulation.
 - c. Peptonization.
 - d. Gas production.
 - e. Presence and character of whey (clear, turbid).

- f. Reaction, decolorization of indicator, etc.
- g. Odor.
- 2. Reincubate (in locker if incubator space is limited) and re-examine after a week or ten days.
- 3. Give the reaction illustrating the formation of lactic acid from lactose.
- 4. Is lactic acid production of any significance in food preservation?
- 5. Are the organisms studied in this exercise of any economic significance? Indicate briefly.

Ref.: Buchanan. *Bacteriology*, pp. 269-280.

EXERCISE 44

BUTYRIC FERMENTATION

A. Materials Needed :

1. Two tubes of dextrose broth.
2. Two tubes of milk.
3. Calcium carbonate.
4. Garden soil.

B. Procedure :

1. To each of the tubes add a pinch of soil and a pinch of CaCO_3 .
2. Heat to 80°C . for ten minutes or bring to a boil over a free flame.
3. Cool quickly.
4. Incubate at room temperature for 48 hours or until the next period.

C. Examination and Record :

1. Record whether there is any characteristic odor and what the odor resembles.
2. Has the milk been coagulated?
3. Describe the character of the curd.
4. Make stained mounts, using Loeffler's methylene blue, and Gram's stain from one tube of milk and one tube of broth, and search for characteristic sporangia of *Cl. butyricum*. Draw.
5. Write the structural formula for butyric acid.

Ref.: Buchanan. *Bacteriology*, pp. 288-289.

EXERCISE 45

PANARY FERMENTATION

A. Materials Needed:

1. Sterile petri dishes containing moistened filter paper and a slice of moistened sterilized bread.
2. Broth culture of *Serratia marcescens*.
3. Broth culture of the bacillus of ropy bread (*B. mesentericus*).

B. Procedure:

1. *To demonstrate the cause of bloody bread:*
Inoculate a slice of bread in a petri dish with a platinum loop of *Serratia marcescens*. Make inoculations in at least a dozen places. Keep in the locker until the next period, preferably 72 hours.
2. *To demonstrate the cause of ropy bread:*
Inoculate a slice of bread in a petri dish with a culture of the bacillus of ropy bread (*B. mesentericus*) and keep in 37° incubator until the next period.

C. Examination and Record:

1. Examine the bread inoculated with *Serratia marcescens*. Is there any pigment? Make a stain of some of the bloody bread; draw.
2. Examine the bread inoculated with the bacillus of ropy bread. Is there any odor? Record appearance, color, etc. Is there any change in consistency of the bread? Make carbol fuchsin stain of some slimy material. Draw, showing characteristic organisms.
3. Is bloody bread commonly encountered? Give an instance, if possible.
4. How may "rope" in bread be prevented?

Ref.: Buchanan. *Bacteriology*, pp. 262-268.

EXERCISE 46

ACETYL-METHYL-CARBINOL PRODUCTION

(Voges-Proskauer Reaction)

A. Materials Needed:

1. Culture of *Esch. coli*.
2. Culture of *Aero. aerogenes*.
3. Culture of *Aero. cloacae*.
4. Four tubes glucose peptone water.
5. NaOH or KOH (10%).
6. KOH (40%), containing 0.3% creatin.

B. Procedure:

1. Inoculate separate tubes of glucose peptone water with the organisms listed.
2. Incubate at 37° C. for 48 hours.

C. Examination and Record:

1. To a portion of each of the cultures and the control add an equal volume of 10% KOH or NaOH, shake, and let stand exposed to the air.
2. To the remaining portion of each of the cultures and control, add an equal volume of the 40% KOH-creatin solution, shake, and let stand exposed to the air.
3. Record whether a pink color develops on the surface of the medium after standing 15 minutes to 2 hours. (If pink color does not develop during the class period, let stand over night and re-examine.)
4. Which test is preferable? Why?

EXERCISE 47

TYPES OF MICROORGANISMS IN VARIOUS FOOD PRODUCTS

A. Materials Needed:

1. Salt rising bread dough.
2. Home made yeast.
3. Pickle brine.
4. Sauerkraut.
5. Silage.

B. Examination and Record:

1. Make stained mounts from each of the above products using methylene blue, or dilute carbol fuchsin, and the Gram stain.
2. Place a drop of each of the above materials on a slide, cover with a thin coverslip and press down to make a film about one-tenth mm. thick. Examine with the high power dry lens to see if yeasts or mold filaments are abundant or rare.
3. List and draw the microorganisms present in each product examined.
4. Which method of staining employed was found to be preferable for demonstrating presence of the organisms?

Ref.: Buchanan. *Bacteriology*, pp. 278-280.

EXERCISE 48

COMPARISON OF ACID AND RENNET CURDS

A. Materials Needed:

1. Two tubes of raw milk (unpasteurized).
2. Rennet solution.
3. N/1 HCl.

B. Procedure:

1. To one tube add normal HCl, a drop at a time until the milk is coagulated.
2. Warm the other tube to about 37° C. and add one-half c.c. rennet solution, a drop at a time. Allow to stand in the warm incubator (or preferably in a water bath at 37° to 39° C.) for 10 or 15 minutes or until coagulation occurs.

C. Examination and Record:

1. Compare the acid and rennet curd as to consistency and general appearance. Are the curds soluble in alkali?
2. How does the acid curd differ from the rennet curd?
3. What is the source of rennet and how may it be prepared?

Ref.: Buchanan. *Bacteriology*, pp. 223, 323.

EXERCISE 49

EFFECT OF STERILIZATION, PASTEURIZATION, AND STORAGE TEMPERATURE ON THE KEEPING QUALITIES OF MILK

It is the object of this exercise to indicate the effect of temperature of heating and incubation on the types of changes occurring in milk.

A. Materials Needed:

1. Clean test tubes.
2. Market milk (preferably unpasteurized raw milk).

B. Procedure:

Each student will tube and plug nine tubes of fresh milk (separated or fat free). Treat these as follows:

1. Place one tube of milk without treatment in the ice chest, one in the cool thermostat, and one in the warm thermostat for 24 to 48 hours.
2. Heat three tubes of milk in a water bath at a temperature of 60° C. for 20 minutes. Cool in water. Distribute in the ice box, cool incubator, and warm incubator, as above.
3. Heat three tubes of milk in boiling water for 20 minutes. Distribute as before.

C. Examination and Record:

1. Record in tabular form the changes which have taken place in the tubes of unheated milk placed in the ice chest, cool thermostat, and warm thermostat using the following as a guide: Reaction, coagulation, peptonization, whey.

2. Examine the tubes of milk that have been pasteurized. Record the same facts as in the preceding.
3. Examine the tubes of milk heated to the boiling point.
 - a. Record as before and compare the results with the preceding.
4. Are the acid formers normally found in milk generally killed by: (a) pasteurization; (b) boiling?
5. How is milk pasteurized?
6. Which of the tubes of milk would you consider still usable?
7. What are the purposes of pasteurization?

Ref.: Buchanan. *Bacteriology*, pp. 225-228, 270.

EXERCISE 50

DECOMPOSITION OF STARCH BY MICRO-ORGANISMS

In this and the three succeeding exercises the rôle and methods of recognizing enzymic action by microorganisms are introduced.

A. Materials Needed:

1. Two petri dishes containing sterile starch agar.
2. Pure culture of yeast. (*Sac. cerevisiae* or other species.)
3. Culture of *Esch. coli*.
4. Culture of *Aero. aerogenes*.
5. Culture of *Aspergillus*.
6. Diastase solution (5%).
7. Lugol's solution (dilute one to three).
8. Starch paste or soluble starch (50 c.c. of a 1/2% solution in a bottle or Erlenmeyer flask).
9. Fehling's solutions.

B. Procedure:

1. By means of a glass pencil mark off each of the petri dishes of starch agar into two sectors or sections.
2. Inoculate one sector by streaking with *Esch. coli*, another with *Aero. aerogenes*, a third with yeast, and the fourth with the mold.
3. Incubate in the locker for two to five days.

C. Examination and Record:

1. Ascertain the action of diastase on starch by proceeding as follows:
 - a. Mix equal volumes of the Fehling's solutions. Bring 5 c.c. of the mixture in a test tube to a boil over the free flame and if no precipitate of copper is formed add 5 drops of the starch paste (a drop

at a time) and continue boiling for 2 or 3 minutes. Record whether or not there was any reduction of Fehling's solution.

- b. To a few c.c. of the starch paste in a test tube add a few drops of Lugol's solution. Record result.
 - c. To the remaining starch paste solution, add about 10 c.c. of diastase and at 15 minute intervals test with Fehling's solution and Lugol's iodine solution as described in "a" and "b" above, until a distinct change is observed with respect to these tests. Record results in tabular form.
2. Flood the petri dish with dilute Lugol's solution; pour off. If the starch has been hydrolyzed, the characteristic iodo-starch reaction will not be observed where hydrolysis has taken place. (A clear zone will be observed in the vicinity of the growth of the microorganisms.) Record results observed.
 3. What are the products of the hydrolysis of starch?
 4. Is diastase an intra- or extra-cellular enzyme?
 5. Of what value is diastase production to microorganisms?

Ref.: Buchanan. *Bacteriology*, pp. 215-219.

EXERCISE 51

DECOMPOSITION OF SUCROSE BY MICRO-ORGANISMS

A. Materials Needed :

1. Five tubes of sucrose (Andrade indicator) peptone water.
2. Fehling's solutions.
3. Culture of *Saccharomyces cerevisiae*.
4. Culture of *Aspergillus*.
5. Culture of *Esch. coli* (*communis*).
6. Culture of *Aero. aerogenes*.
7. Sucrose, glucose, and levulose solutions (1.0%) in water.

B. Procedure :

1. Inoculate each of the cultures given above into separate tubes of sucrose peptone solution.
2. Incubate at room temperature, 48 to 72 hours.

C. Examination and Record :

1. Note any change in reaction.
2. Test portions of each of the inoculated tubes with Fehling's solution as directed in Exercise 49. (Tabulate.)
3. Test solutions of sucrose, glucose, and levulose with Fehling's solution. (Tabulate.)
4. Write the reaction for the inversion of cane sugar.
5. What enzyme is responsible for the breaking up of sucrose into glucose and levulose?
6. Define the term enzyme.

EXERCISE 52

DECOMPOSITION OF GELATIN BY MICRO-ORGANISMS

A. Materials Needed:

1. Culture of *Esch. coli* in broth.
2. Culture of *Proteus vulgaris* or *Ser. marcescens* in broth.
3. Culture of *B. subtilis* or *Flavo. suaveolens* in broth.
4. Culture of *Saccharomyces cerevisiae* in wort or yeast water or malt extract broth.
5. Culture of *Aspergillus*.
6. Six tubes of nutrient gelatin containing about 0.02% glucose.
7. Six petri dishes with Frazier's gelatin medium.
8. Tannic acid (1% solution).
9. Mercuric chloride solution (HgCl_2 , 15 g.; conc. HCl , 20 c.c.; water, 100 c.c.).

B. Procedure:

1. Inoculate a tube of gelatin with *Esch. coli* by spreading several loops of the culture (or a drop) over the surface of the gelatin.
2. Repeat with each of the other organisms. (The mold spores merely need be sprinkled on the surface of the gelatin.)
3. Incubate at 20° C. for two days to a week.
4. With a glass pencil divide each of the petri dishes into two sectors.
5. Inoculate a sector, on each of two plates, with one of the cultures by placing a loop in the middle of the medium. (This leaves one sector as a control.)

Note: Employ *Esch. coli* or *Sacch. cerevisiae* and any two of the other organisms.

6. Incubate at 20° C. for 48 hours.

C. Examination and Record:

1. Examine the tubes and record the relative amount of liquefaction of gelatin by the various microorganisms after two days and after one week.
2. Flood a petri dish containing the giant colonies of the test organisms with tannic acid, and the duplicate dish with the mercuric chloride solution, and let stand for about half an hour.
3. Record appearance of medium in vicinity of colony.

Note: (A large clear zone around colony on plate flooded with HgCl_2 , appearing in 15 to 30 minutes, and a clear zone around colony with precipitate at the edge of this clear zone appearing immediately on the plate flooded with tannic acid, indicates a strong reaction).

4. Define protease.
5. Is gelatinase an intra- or extra-cellular enzyme? Of what value is it to the microorganisms?

Ref.: Buchanan. *Bacteriology*, pp. 209-210, 219-220.

EXERCISE 53

TO ILLUSTRATE THE RÔLE OF YEAST AND DIASTASE IN BREAD MAKING

A. Materials Needed:

1. Two Smith fermentation tubes.
2. Solution of diastase.
3. Pure culture of *Saccharomyces cerevisiae* (or compressed yeast).
4. 100 c.c. of 2% starch in tap water containing 1.0 g. NH_4Cl , 1.0 g. K_2HPO_4 and 5.0 c.c. N/1 citric acid per liter.

B. Procedure:

1. Fill two Smith tubes with the starch medium. To one of them add 2 c.c. of the diastase solution and let stand for one hour. (Warm to 37°C . and keep warm.)
2. Sterilize both tubes in the autoclave at 10 pounds for 10 minutes or in the Arnold for 30 minutes (make sure that the closed arm of the Smith tube is full before sterilization).
3. Inoculate each of the Smith tubes (after cooling) with the pure culture of *Sac. cerevisiae* (or yeast cake).¹
4. Incubate in the locker until the next period.

C. Examination and Record:

1. Determine the reaction to litmus or brom-thymol-blue and record.
2. Record whether gas was formed.
3. Explain the results obtained.
4. Is there any analogy between this experiment and the reactions taking place in the rising of bread dough? Explain.

Ref.: Buchanan. *Bacteriology*, pp. 262-268.

¹ If yeast cake is used, inoculation should be made with an inner portion obtained aseptically, as contamination with bacteria will vitiate the results.

EXERCISE 54

EFFECT OF DIRECT SUNLIGHT ON BACTERIA

It is the purpose of this exercise not merely to demonstrate the germicidal action of sunlight, but also to introduce the technique of smearing plates which is frequently employed for the isolation of intestinal pathogens and in the bacterial examination of water.

A. Materials Needed :

1. Culture of *Esch. coli* (24 hour broth cultures).
2. Three tubes of agar for plates (1/2 to 2/3 full).
3. Tube of physiological salt solution.
4. Sterile petri dishes (3).
5. Glass rod for smearing plates.

(Consists of a seven inch glass rod bent one and one-half or two inches from one end at an angle of about 105 degrees. This rod is sterilized by passing the short end through the flame several times.)

B. Procedure :

1. Melt the agar, pour into plates, and allow to harden.
2. Inoculate the tube of salt solution with a small loop of the broth culture of *Esch. coli*.
3. Place a loop of the salt solution dilution in the center of each plate and smear it over the surface of the agar with the flamed glass rod.
4. Expose the plates to direct sunlight (outdoors), for 15-30 minutes, leaving the lid of one off, another on, and covering the third dish with a piece of black paper or cloth.
5. Incubate at 37° C. for 24 hours.

C. Examination and Record :

1. Examine the plates and record the relative abundance and vigor of growth of colon colonies and explain results.

2. Which rays are most germicidal?
3. Is disinfection by light employed practically? If so, give examples.
4. Is the method of smearing a culture over the surface of solid media applicable to (1) the study of colony characteristics, and (2) isolation of pure cultures? Why?

Ref.: Buchanan. *Bacteriology*, pp. 168-169.

EXERCISE 55

EFFECT OF REACTION ON GROWTH OF MICROORGANISMS ¹

In this exercise is introduced a method for obtaining acidities and alkalinities which are too great for sterilization.

A. Materials Needed:

1. Approximately 5N citric acid solution.
2. Approximately N/1 NaOH solution.
3. Twenty-eight sterile petri dishes.
4. Sterile 1.0 c.c. pipettes graduated in 1/10 c.c.
5. Seven bottles or flasks each containing 50 c.c. of glucose phosphate agar. (1% peptone, 1% glucose, 0.2% K_2HPO_4 .)
6. Broth cultures of *Esch. coli* and *B. subtilis*.
7. Wort or malt extract broth cultures of *Sac. cerevisiae* and *Torula rosea*.
8. Agar cultures of *Aspergillus* and *Penicillium* or other molds.

B. Procedure:

1. Number the bottles of agar from 1 to 7, melt and cool to about 45° C.
2. Add the quantities of 5N acetic or N/1 NaOH as indicated below; pour four plates from each bottle and allow to cool and solidify.

Series	Bottle # 1	5.0 c.c. 5N Citric acid
"	" # 2	2.0 c.c. " " "
"	" # 3	0.5 c.c. " " "
"	" # 4	No addition
"	" # 5	0.5 c.c. N/1 NaOH
"	" # 6	1.0 c.c. " "
"	" # 7	3.0 c.c. " "

¹ It is suggested that two or three students work together on this exercise because of the large amount of material needed.

This gives 7 series (each of 4 agar plates) ranging in reaction from strongly acid through neutral to strongly alkaline.

3. Inoculate one plate of each series with the culture of *Esch. coli* and *B. subtilis* as follows:
 - a. Mark the bottom of the petri dish so as to divide it approximately into halves.
 - b. Dip a small platinum loop into the culture of *Esch. coli*, then transfer the loopful of the culture to the plate by touching the loop to the surface of the solidified agar medium. Transfer 3 or 4 loops as indicated above into half of the plate.
 - c. Transfer 3 or 4 loops of the culture of *B. subtilis* to the remaining half of each plate.
4. Inoculate one plate of each series with the culture of *Sac. cerevisiae* and *Torula rosea* as described in B-3 above.
5. Inoculate one plate of each series with the two molds. This may be accomplished as follows:

Heat the needle or loop and while still hot touch the solid medium containing the mold culture. Then touch this sticky needle to the mold growth and transfer the adhering spores to the desired agar plates as was done in B-3 above.
6. Leave the remaining plates of each series exposed to the air for 15 minutes.

C. Examination and Record:

1. Record in tabular form the relative vigor of growth of the various pure cultures employed. Indicate the average diameter of the colonies on the different media.
2. For the plates exposed to the air, record.
 - a. Number of colonies on each plate.
 - b. Nature of colonies (whether bacteria, yeast, or molds).
3. Discuss briefly relation of reaction to growth of bacteria, yeasts, and molds. (Express reactions in terms of per cent normal acid or alkali.)

EXERCISE 56

EFFECT OF DESICCATION ON BACTERIA

A. Materials Needed:

1. Broth culture of *Esch. coli*.
2. Broth culture of *B. subtilis*.
3. Sterile petri dishes.
4. Sterile glass beads (10 to 15) in test tubes (2).
5. Forceps or stiff platinum loop.
6. Nutrient agar in test tubes.
7. Pipettes.

B. Procedure:

1. Cover the beads in one of the test tubes with 2 or 3 c.c. of the culture of *Esch. coli*, shake thoroughly, pour off excess culture (take care to thoroughly flame the mouth of the tube), and then pour the impregnated beads into a sterile petri dish.
2. Cover the beads in the remaining test tube with the culture of *B. subtilis* and proceed as directed for *Esch. coli* above.
3. Incubate the petri dishes at 37° C.
4. At convenient times (every few days, or every laboratory period) remove one of the *Esch. coli* and one of *B. subtilis* impregnated beads (by means of sterile forceps or loop) to separate sterile petri dishes and add a tube of nutrient agar. Incubate at 37° C. for a day or two and record whether typical *coli* or *subtilis* colonies are present. (This procedure should be continued until at least one of the organisms has died off.)

C. Examination and Record:

1. Record the viability of *Esch. coli* in days.
2. Record the viability of *B. subtilis* in days.

3. Explain the results obtained.
4. Discuss briefly, transmission of disease through the air as influenced by desiccation. (Pathogenic bacteria are generally non-spore formers.)

Ref.: Buchanan. *Bacteriology*, pp. 478-479.

EXERCISE 57

TO DEMONSTRATE PROPERTIES OF AN ANTI-SEPTIC AND DISINFECTANT

A. Materials Needed:

1. Three glucose broth fermentation tubes marked 1b, 2b, and 3b.
2. One tube of nutrient broth containing 0.05% phenol marked 1a.
3. One tube of 0.3% phenol broth marked 2a.
4. One tube of 1.0% phenol broth marked 3a.
5. Young culture (24 hour broth) of *Esch. coli*.

B. Procedure:

1. Inoculate tubes 1a, 2a, and 3a with a standard loop of the broth culture of *Esch. coli*. (Special care must be taken not to touch the sides of the tubes.) Allow to stand in the room for one hour.
2. Transfer one loop from tube 1a to 1b, from 2a to 2b, and from 3a to 3b.
3. Incubate all six tubes at the body temperature for 48 hours or until the next period.

C. Examination and Record:

1. Examine the six tubes of broth and record in tabular form whether gas and growth are present.
2. Define antiseptic and disinfectant.
3. Does this experiment illustrate these terms?
4. Name three antiseptics and disinfectants of each of the following classes: (a) gaseous, (b) metallic, (c) organic, (d) oxidizing, (e) coal tar products.

Ref.: Buchanan. *Bacteriology*, pp. 183-193.

EXERCISE 58

ANTISEPTIC ACTION OF VARIOUS MATERIALS

A. Materials Needed:

1. Broth or agar culture of *Esch. coli*, *Staph. aureus*, or *Aero. aerogenes*.
2. Four tubes of melted agar.
3. Four petri dishes.
4. Zinc oxide tape, corn plaster, copper coin, silver coin, zinc oxide salve, carbolated vaseline, bits of filter paper dipped in eucalyptus oil, mercurochrome or other test antiseptic or disinfectant materials.
5. Sterile broth in tubes.

B. Procedure and Record:

1. Inoculate a tube of clear melted agar (at not over 45° C.) with several loops of one of the organisms and pour into a petri dish. Prepare all dishes in this manner.
2. After the agar has cooled place at various points on its surface one or more of the following materials:
 - a. Corn or bunion plaster.
 - b. Small strip of zinc oxide adhesive tape.
 - c. A copper coin (clean).
 - d. A silver coin (clean).
 - e. A bit of zinc oxide salve.
 - f. A bit of carbolated vaseline.
 - g. A piece of paper soaked in mercurochrome or other test material.
3. Place in 37° C. incubator for 24 to 48 hours.
4. Record any evidence of inhibition of growth.
5. Remove a bit of the agar in the clear zone (if present) and inoculate into broth to ascertain whether growth was merely inhibited or the organisms killed.
6. Place dishes in the locker and re-examine after one week. Record any differences.

EXERCISE 59

SELECTIVE ACTION OF GENTIAN VIOLET ON GROWTH OF BACTERIA

A. Materials Needed:

1. Broth cultures of *Esch. coli*, *Aero. aerogenes*, *Serr. marcescens*, *B. subtilis*, *Staph. aureus*, *Sarc. lutea*.
2. Four tubes of agar. (Approximately 10 c.c.)
3. Four sterile petri dishes.
4. Sterile 1 c.c. pipettes.
5. Solution of sterile (1 to 10,000 dilution) gentian violet or preferably crystal violet (85% dye).

B. Procedure:

1. Pour a tube of melted agar into each of two petri dishes.
2. To each of the two remaining tubes of melted agar add 1 c.c. of 1 to 10,000 dilution of the dye. (This makes approximately a dilution of 1 to 100,000 of gentian or crystal violet.) Pour these tubes of agar into the two remaining sterile dishes and allow to solidify.
3. Using any four of the cultures listed above, streak each on to agar with and without gentian violet. (Two organisms may be streaked on one plate.)
4. Incubate at 37° C. until the next period.

C. Examination and Record:

1. Record in tabular form the Gram stain and vigor of growth of the respective organisms.
2. Discuss briefly the significance of these results.

EXERCISE 60

OSMOTIC PRESSURE AND FOOD PRESERVATION

A. Materials Needed :

1. Sweetened condensed milk.
2. Tap water and 5%, 10%, 15%, 20%, and 25% NaCl in tap water.
3. Fresh beef cut in small dice.
4. Clean test tubes.

B. Procedure :

1. Influence of salt concentration.

- a. Cut a piece of beef into small dice. Place one piece in each of seven tubes. To these tubes add as follows:

Tube No. 1	5 c.c. tap water
Tube No. 2	5 c.c. 5% NaCl
Tube No. 3	5 c.c. 10% NaCl
Tube No. 4	5 c.c. 15% NaCl
Tube No. 5	5 c.c. 20% NaCl
Tube No. 6	5 c.c. 25% NaCl

- b. Plug with cotton and keep in the warm thermostat until the next period (preferably not more than 48 hours). Be sure that the meat is entirely immersed.

2. Influence of concentration of sugar.

- a. Place dilutions of sweetened condensed milk in test tubes as follows:

Tube No. 1.	Condensed milk = concentrated.
Tube No. 2.	Milk 2 pt. + H ₂ O 1 pt. = 66% concentration.
Tube No. 3.	Milk 1 pt. + H ₂ O 1 pt. = 50% concentration.
Tube No. 4.	Milk 1 pt. + H ₂ O 2 pt. = 33% concentration.
Tube No. 5.	Milk 1 pt. + H ₂ O 3 pt. = 25% concentration.

- b. Plug with cotton and keep in the locker for 48 hours or until the next period (preferably 48 hours).

C. Examination and Record:

1. Examine the tubes of meat and record the relative amount of growth in each tube as indicated by odor and clouding. (Tabulate results.)
2. Discuss the relation of salt concentration and meat preservation. Is this of any practical significance?
3. Compare and record the appearance and odor of the various dilutions of condensed milk. (Tabulate results.)
4. Define plasmolysis.
5. Which exerts a greater osmotic pressure, 25% glucose or 25% NaCl? Give reasons for your answer.

Ref.: Buchanan. *Bacteriology*, pp. 166-168.

EXERCISE 61

TO ILLUSTRATE DISINFECTING ACTION OF METABOLIC END PRODUCTS (ACIDS)

A. Materials Needed:

1. Six tubes of glucose peptone solution (1% glucose, 1% peptone) in Durham fermentation tubes.
2. Young culture of *Esch. coli*.
3. Young culture of *Aero. aerogenes*.
4. Young culture of *Aero. cloacae*.
5. Three tubes of glucose broth.
6. Methyl red indicator.

B. Procedure

1. Inoculate two tubes of glucose peptone solution with *Esch. coli*, two with *Aero. aerogenes*, and two with *Aero. cloacae*.
2. Incubate at 37° C.

C. Examination and Record:

1. After 24 to 48 hours record presence of gas and growth. Add to **one** tube of each of the organisms about 1/2 c.c. of methyl red, record reaction and discard. Leave the remaining tube of each organism in the 37° C. incubator for two weeks.
2. Make transfers from the two week old cultures of *Esch. coli*, etc., to glucose broth, incubate at 37° C. for 24–48 hours and record whether typical growth has occurred.
3. Add about 1/2 c.c. of methyl red to each of the two week old cultures and note the reaction.
4. Explain results.

EXERCISE 62

THERMAL DEATH POINT

(*Esch. coli*)

A. Materials Needed:

1. Broth culture of *Esch. coli* (24 hours).
2. Seven test tubes containing equal quantities of broth.
3. Water bath.
4. Thermometer.

B. Procedure:

1. Put all the tubes of broth in the water bath. (The water in the bath should be above the height of the broth in the tubes.) In one tube place a thermometer, then raise the temperature of the bath slowly to 50° C.
2. Inoculate a tube (one only) of broth with a loop of the young culture of *Esch. coli*, taking particular care not to touch the side of the tube.
3. Keep the temperature constant at 50° C. for ten minutes, then remove the inoculated tube to a can of cold tap water and label with the temperature of exposure.
4. Raise the temperature of the bath to 55° C. Inoculate a second tube, and after ten minutes' exposure, remove it, cool, and label as directed above.
5. In the same manner expose *Esch. coli* to temperatures of 60° C., 65° C., 70° C., and 75° C.
6. Incubate all of the cooled tubes at 37° C. until the next period.

C. Examination and Record:

1. Record whether typical growth has occurred in the various tubes.

2. Taking the true thermal death point of *Esch. coli* as 58° C. for 10 minutes, how do your results agree with this and what is a possible explanation of the discrepancy noted, if any?
3. Define thermal death point.
4. Is the determination of this point of any practical significance in the industries or in the home? If so, illustrate with examples.
5. List the more important factors which influence the thermal death point of a microorganism.

Ref.: Buchanan. *Bacteriology*, pp. 169-174.

EXERCISE 63

EFFECT OF REACTION ON FOOD PRESERVATION

A. Materials Needed:

1. Apple (not over-ripe).
2. Dried peas.
3. Methyl red, brom-thymol-blue, and phenol red indicators.
4. Eight sterile test tubes.

B. Procedure:

1. Cut small dice from an apple and fill four test tubes one-third full. Cover with tap water.
2. Place about a dozen small peas in each of four test tubes and cover with water. (Use sufficient water to allow for absorption.)
3. Heat two tubes of apple and two of peas in boiling water or in the Arnold for 20 minutes.
4. Incubate one tube of heated and one of unheated apples and peas at 37° C. until the next period and proceed with the remaining tubes as directed below.

C. Examination and Record:

1. Divide one of the heated tubes of apple (the one not incubated) into three parts, add a few drops of methyl red to one part, brom-thymol-blue to another, and phenol red to the third. Record acidity or alkalinity to each indicator.
2. Determine the reaction of the heated tube of peas and the unheated tube of peas and apples as directed for C-1 above. Record all results in tabular form.

3. Record appearance and whether growth has occurred in any of the incubated tubes.
4. How is the effect of H^+ ion concentration on sterilization indicated in this experiment?

Ref.: Buchanan. *Bacteriology*, pp. 173-174, 234-236.

EXERCISE 64

BACTERIA OF THE AIR

A. Materials Needed:

1. Three tubes of nutrient agar (two-thirds full).
2. Three sterile petri dishes.

B. Procedure:

1. Melt three tubes of agar, pour each into a sterile petri dish and allow the agar to harden.
2. Expose each dish in a different place by removing the cover and allowing the dish to stand open for 15 minutes. One may be infected by sneezing or coughing violently above it or by shaking a dusty rag into it, etc.
3. Keep these in the cool or gelatin thermostat or locker for several days but not more than one week.

C Examination and Record:

1. Count and record the number of bacterial and mold colonies that have been formed.
2. Compute the number of bacteria falling per square foot per hour in each of the places in which plates were exposed.
3. Record the number of varieties of bacteria present as indicated by differences in gross appearance of colonies.
4. Draw and describe three colonies of different kinds. Use the directions given in Exercise 33 as a model for the description.
5. Prepare mounts (using Gram's stain) from each of the three colonies used in 4 above. Draw and describe each of these organisms. (See Exercises 11 and 12 for characteristics to be noted.)

Ref.: Buchanan. *Bacteriology*, pp. 477-482.

EXERCISE 65

STUDY OF PIGMENT FORMATION

A. Materials Needed :

1. Agar slant culture of *Serratia marcescens*.
2. Agar slant culture of *Ps. aeruginosa* (*pyocyanea*).
3. Agar slant culture of *Staph. aureus*.
4. Agar slant culture of *Sarcina lutea* or *Micrococcus luteus*.
5. Agar slants (four tubes).
6. Gelatin (four tubes).
7. Potato (four tubes).
8. Broth (four tubes).

B. Procedure :

1. Inoculate each culture into gelatin, potato, agar, and broth.
2. Place gelatin in the 20° C. incubator and other cultures in the locker.
3. Incubate for one week.

C. Examination and Record :

1. Examine broth, agar, gelatin, and potato. Record presence, distribution, and color of pigment.
2. Is pigment production by bacteria of any significance in the industries? Cite examples.

Ref.: Buchanan. *Bacteriology*, pp. 318-319.

EXERCISE 66

THE DESCRIPTIVE CHART FOR THE IDENTIFICATION OF BACTERIA

The purpose of this exercise is threefold:

1. to acquaint the student with the official chart of the Society of American Bacteriologists for the description of bacteria,
2. to familiarize the student with the use of keys for the identification of bacterial species,
3. to test the ability of the student to apply what he should have learned in previous exercises.

A. Materials Needed:

1. Descriptive charts of the Society of American Bacteriologists.
2. An unknown mixture of bacteria in broth or in salt solution.
3. Various standard media to be ascertained and called for by the student as the isolation and identification of the unknown organism progresses.

B. Procedure:

1. Isolate the different organisms present in the mixture supplied, on to agar slants.
2. Ascertain the morphology, cultural and physiological characteristics, of the isolated organisms.

C. Examination and Record:

1. On a descriptive chart (S.A.B.) summarize all data which you have secured for *Esch. coli*.
2. On another descriptive chart summarize the data for *B. subtilis*.
3. Make a brief characterization of *Esch. coli* and *B. subtilis* and determine their index numbers.
4. Record the characteristics of your unknown organisms

on separate descriptive charts and determine their index numbers.

5. Ascertain the genus and species of your unknowns with the aid of Bergey's *Determinative Bacteriology*, if the accompanying partial key is inadequate.

The following key is presented as an aid to introducing the student into the field of systematic bacteriology. The differential characteristics employed are necessarily restricted to those with which the elementary student is already familiar or which he may readily utilize. Pathogenicity, serological reactions, complex chemical determinations, such as quantitative estimation of nitrogen or specific organic acids, are considered beyond the beginner, at this stage of his development, and therefore not feasible as differential characters. The scope of the appended key is consequently seriously restricted. Because of the frequent references in the classroom to some of the more common pathogenic forms, the acetic acid producing, nitrogen fixing and nitrifying, organisms, a few species from these groups are included as illustrative of their relationships in a systematic arrangement of the bacteria.

No pretense is made to completeness. A number of species was chosen from each of the genera considered as illustrative of feasible and possible species differentiation within the respective genera. Bergey's *Determinative Bacteriology*, Buchanan's *General Bacteriology*, and the author's studies on the intestinal groups of bacteria were drawn upon for selection of differential characteristics.

The key will be found most useful if employed with the foregoing limitations in mind, its use for instructional purposes being restricted to organisms falling within its restricted scope and not requiring determination of pathogenicity, nitrogen fixation, nitrification, or acetic acid production for their differentiation. It must not be employed as a substitute for the more pretentious and exhaustive contributions on determinative bacteriology but rather as an introduction to such works.

I. KEY TO SOME OF THE MORE COMMONLY ENCOUNTERED FAMILIES OF BACTERIA

A. Acid fast or possessing metachromatic granules (may show branching or other mold-like characters).

I. *Mycobacteriaceae*.

AA. Neither acid fast nor possessing metachromatic granules (do not show branching or other mold-like characters).

B. Do not grow in media containing organic matter (oxidize ammonia to nitrites, or nitrites to nitrates for growth energy).

II. *Nitrobacteriaceae*.

BB. Generally grow well in media containing organic matter (do not oxidize ammonia to nitrites nor nitrites to nitrates for growth energy).

C. Cells spherical.

III. *Coccaceae*.

CC. Cells not spherical.

D. Cells straight rods.

E. Endospores produced.

IV. *Bacillaceae*.

EE. Endospores not produced.

V. *Bacteriaceae*.

DD. Cells bent or spiral rods.

VI. *Spirillaceae*.

II. KEY TO SOME GENERA AND SPECIES OF THE FAMILY MYCOBACTERIACEAE

A. Acid fast.

B. Grow very poorly at room temperature (pathogenic for man, animals, or birds).

C. Grow poorly at 42° C.

D. Slender rods, yellowish or reddish growth on glycerol agar; permanent acidity from glycerol.

1. *Mycobacterium tuberculosis*.
(hominis)

DD. Plump rods, grayish growth on glycerol agar; initial acidity from glycerol followed by alkalinity.

2. *Mycobacterium tuberculosis*.
(bovis)

CC. Plump rods, good growth at 42° C.

3. *Mycobacterium avium*.

BB. Grow moderately well at room temperature. (Not pathogenic, may be parasitic.)

C. Litmus milk reduced and coagulated; yellow growth on potato.

4. *Mycobacterium smegmatis*.

CC. Litmus not reduced; milk not coagulated.

D. Litmus milk alkaline.

5. *Mycobacterium butyricum*.

DD. Litmus milk becomes dirty yellowish brown.

6. *Mycobacterium friburgensis*.

II. KEY TO SOME GENERA AND SPECIES OF THE FAMILY MYCOBACTERIACEAE—Continued

AA. Not acid fast (have metachromatic granules).

B. Cells slender, curved, or straight, generally club-shaped with distinct granules; produce toxin.

7. *Corynebacterium diphtheriae*.

BB. Cells thick, solid, barred, wedge-shaped forms, etc. Do not produce toxin.

C. Growth vigorous on blood serum.

D. Granules small, growth glistening, yellow to salmon pink; acid produced from sucrose.

8. *Corynebacterium hoagii*.

DD. Granules large, growth dry, white to yellow; acid not produced from sucrose.

9. *Corynebacterium flavidum*.

CC. Growth poor on blood serum.

D. Aerobic; acid produced from sucrose.

10. *Corynebacterium xerosis*.

DD. Anaerobic or microaerophilic; acid not produced from sucrose; cells wedge-shaped.

11. *Corynebacterium acne*.

III. KEY TO SOME GENERA AND SPECIES OF THE FAMILY NITROBACTERIACEAE

A. Oxidize ammonia to nitrites.

B. Cells rod-shaped.

C. Motile.

CC. Non-motile.

BB. Cells spherical.

AA. Oxidize nitrites to nitrates.

B. Gram positive; indol negative.

BB. Gram negative; indol positive.

1. *Nitrosomonas europaea*.

2. *Nitrosomonas groningensis*.

3. *Nitrosococcus nitrosus*.

4. *Nitrobacter punctatus*.

5. *Nitrobacter flavus*.

IV. KEY TO SOME GENERA AND SPECIES OF THE FAMILY COCCACEAE

- A. Cells typically in pairs.
 - B. Gram positive (usually capsulated).
 - BB. Gram negative (usually not capsulated).
 - C. Acid from dextrose.
 - DD. No acid from maltose.
 - CC. No acid from dextrose.
 - D. Acid from maltose.
- AA. Cells not typically in pairs.
 - B. Cells typically in chains.
 - C. Blood agar neither hemolyzed nor greened.
 - D. Casein peptonized.
 - DD. Casein not peptonized.
 - E. Litmus milk acid and curdled.
 - F. Litmus reduced; gas not produced.
 - FF. Litmus not reduced (except slightly at bottom of tube); gas produced.
 - EE. Litmus milk not sufficiently acidified to curdle.
 - 7. *Streptococcus paracitronus*.
 - 8. *Streptococcus citronus*.
 - D. Casein not peptonized.
 - C. Blood agar neither hemolyzed nor greened.
 - D. Casein peptonized.
 - DD. Casein not peptonized.

1. *Diplococcus pneumoniae*.

2. *Neisseria meningitidis*.

3. *Neisseria gonorrhoeae*.

4. *Neisseria catarrhalis*.

5. *Streptococcus liquefaciens*.

6. *Streptococcus lactis*.

7. *Streptococcus paracitronus*.

8. *Streptococcus citronus*.

IV. KEY TO SOME GENERA AND SPECIES OF THE FAMILY COCCACEAE—Continued

- CC. Blood agar hemolyzed or greened.
- D. Blood hemolyzed; medium not greened.
- E. Acid from lactose.
- EE. No acid from lactose.
- DD. Blood not hemolyzed; medium greened.
- E. Acid from mannitol.
- EE. No acid from mannitol.
- BB. Cells not in chains.
- C. Cells in irregular groups.
- D. Gram positive.
- E. Pigment not produced on agar.
- F. Nitrates reduced.
- G. Gelatin liquefied.
- GG. Gelatin not liquefied.
- FF. Nitrates not reduced.
- G. Gelatin liquefied.
- GG. Gelatin not liquefied.
13. *Staphylococcus albus*.
14. *Staphylococcus epidermidis*.
15. *Staphylococcus ureae*.
16. *Staphylococcus candidus*.
9. *Streptococcus pyogenes*.
10. *Streptococcus equi*.
11. *Streptococcus faecalis*.
12. *Streptococcus mitior*.

IV. KEY TO SOME GENERA AND SPECIES OF THE FAMILY COCCACEAE—Continued

EE. Pigment produced on agar.

F. Orange pigment on agar.

FF. Yellow pigment on agar.

G. Nitrates reduced.

GG. Nitrates not reduced.

H. Gelatin liquefied.

HH. Gelatin not liquefied.

FFF. Red pigment on agar.

G. Gelatin liquefied.

GG. Gelatin not liquefied.

H. Motile.

HH. Non-motile.

DD. Gram negative.

CC. Cells in regular groups (cubes).

D. Gelatin liquefied; litmus milk not acidified.

DD. Gelatin not liquefied; litmus milk acid and coagulated.

17. *Staphylococcus aureus*.

18. *Staphylococcus citreus*.

19. *Staphylococcus flammus*.

20. *Staphylococcus luteus*.

21. *Rhodococcus roseus*.

22. *Rhodococcus agilis*.

23. *Rhodococcus rhodochrous*.

24. *Micrococcus sphaeroides*.

25. *Sarcina lutea*.

26. *Sarcina lactea*.

V. KEY TO SOME GENERA AND SPECIES OF THE FAMILY BACILLACEAE

A. Aerobic and facultative.

B. Gas produced from lactose and dextrose.

BB. Gas not produced from lactose nor dextrose.

C. Motile.

D. Spores central.

E. Pigment not formed on agar.

F. Gelatin liquefied.

G. Litmus milk peptonized; acid not produced.

H. Nitrates reduced.

I. Starch hydrolyzed; blood serum liquefied.

2. *Bacillus subtilis*.

II. Starch not hydrolyzed; blood serum not liquefied.

3. *Bacillus mycoides*.

HH. Nitrates not reduced.

I. Starch hydrolyzed.

4. *Bacillus megatherium*.

II. Starch not hydrolyzed.

5. *Bacillus cereus*.

GG. Litmus milk not peptonized; acid.

6. *Bacillus lactis*.

FF. Gelatin not liquefied.

1. *Aerobacillus acetoethylicus*.

V. KEY TO SOME GENERA AND SPECIES OF THE FAMILY BACILLACEAE—Continued

- G. Nitrates reduced; thermophilic. 7. *Bacillus thermoamylolyticus*.
- GG. Nitrates not reduced; mesophilic. 8. *Bacillus graveolens*.
- EE. Brownish, yellowish, or creamy white on agar.
 - F. Litmus milk peptonized.
 - G. Litmus milk coagulated. 9. *Bacillus vulgatus*.
 - GG. Litmus milk not coagulated.
 - H. Acid from dextrose and sucrose. 10. *Bacillus mesentericus*.
 - HH. No acid from dextrose or sucrose.
 - 11. *Bacillus fusiformis*.
 - 12. *Bacillus centrosporus*.
 - FF. Litmus milk not peptonized.
 - DD. Spores terminal or subterminal.
 - E. Pigment not formed on agar.
 - F. Gelatin liquefied; acid not produced from carbohydrates. 13. *Bacillus terminalis*.
 - FF. Gelatin not liquefied; acid from dextrose, lactose, and sucrose. 14. *Bacillus circulans*.
 - EE. Yellowish on agar. 15. *Bacillus lactimorbus*.
 - CC. Non-motile.

V. KEY TO SOME GENERA AND SPECIES OF THE FAMILY BACILLACEAE—Continued

D. Nitrates reduced; litmus milk alkaline, not coagulated; ends of cells rounded.

16. *Bacillus panis*.

DD. Nitrates not reduced; litmus milk acid and coagulated; ends of cells truncate.

17. *Bacillus anthracis*.

AA. Anaerobic.

B. Spores central.

C. Motile.

D. Gelatin liquefied.

E. Blood serum liquefied; brain medium blackened.

F. Glycerol fermented with acid and gas.

G. Litmus milk coagulated; neither acid nor gas from sucrose.

18. *Clostridium sporogenes*.

GG. Litmus milk not coagulated; acid from sucrose.

19. *Clostridium botulinum*.

FF. Neither acid nor gas from glycerol. 20. *Clostridium aerofetidum*.

EE. Blood serum not liquefied; brain medium not blackened.

21. *Clostridium chauvoei*.

DD. Gelatin not liquefied.

E. Diameter of spores greater than that of vegetative cells.

22. *Clostridium multifementans*.

V. KEY TO SOME GENERA AND SPECIES OF THE FAMILY BACILLACEAE—Continued

- EE. Diameter of spores less than that of vegetative cells.
 - 23. *Clostridium butyricum*.
- CC. Non-motile.
 - D. Sucrose and lactose fermented with acid and gas; litmus milk acid, coagulated.
 - 24. *Clostridium welchii*.
 - DD. Neither acid nor gas from sucrose nor lactose; litmus milk slowly coagulated and peptonized.
 - 25. *Clostridium bifermentans*.
- BB. Spores terminal.
 - C. Motile; gelatin liquefied.
 - D. Brain medium blackened.
 - E. Litmus milk slightly alkaline; casein not digested.
 - 26. *Clostridium tetani*.
 - EE. Litmus milk rose or yellow colored; casein digested.
 - 27. *Clostridium putrificum*.
 - DD. Brain medium not blackened.
 - 28. *Clostridium tetanomorphum*.
- CC. Non-motile.
 - D. Acid and gas from dextrose; mesophilic.
 - 29. *Clostridium filiformis*.
 - DD. Neither acid nor gas from dextrose; thermophilic.
 - 30. *Clostridium thermoputrificum*.

VI. KEY TO SOME GENERA OF THE FAMILY BACTERIACEAE

A. Utilize (fix) atmospheric nitrogen aerobically.

B. Symbiotic (usually found in the nodules on roots of leguminous plants). I. *Rhizobium*.

BB. Free living in soil; non-symbiotic.

II. *Azotobacter*.

AA. Do not utilize (fix) atmospheric nitrogen.

B. Oxidize alcohol to acetic acid.

III. *Acetobacter*.

BB. Do not oxidize alcohol to acetic acid.

C. Produce water soluble green, blue, or yellowish-green pigment (generally motile by means of polar flagella).

IV. *Pseudomonas*.

CC. Do not produce water soluble pigment as above (flagella, if present, peritrichous).

D. Chromogenic on nutrient agar.

E. Pigment red.

V. *Serratia*.

EE. Pigment violet.

VI. *Chromobacterium*.

EEE. Pigment yellow.

VII. *Flavobacterium*.

DD. Not chromogenic on agar.

E. Gram positive.

VIII. *Lactobacillus*.

EE. Gram negative.

F. Produce gas from lactose.

VI. KEY TO SOME GENERA OF THE FAMILY BACTERIACEAE—Continued

G. Produce acetoin (acetyl methyl carbinol) from dextrose.

IX. Aerobacter.

GG. Do not produce acetoin from dextrose.

H. Grow luxuriantly on citric acid as a sole source of carbon, with development of alkaline reaction. **X. Citrobacter.**

HH. Grow very poorly if at all on citric acid as sole source of carbon; reaction not appreciably changed.

XI. Escherichia.

FF. Gas not produced from lactose.

G. Produce gas from sucrose.

XII. Proteus.

GG. Gas not produced from sucrose.

H. Gas produced from dextrose.

XIII. Salmonella.

HH. Gas not produced from dextrose.

I. Acid formed from dextrose.

J. Motile.

XIV. Eberthella.

JJ. Non-motile.

XV. Shigella.

II. Acid not formed from dextrose. **XVI. Alcaligenes.**

VII. KEY TO SOME SPECIES OF NITROGEN FIXING BACTERIA OF THE FAMILY
BACTERIACEAE

(The genera *Azotobacter* and *Rhizobium*)

A. Free living; do not produce nodules on roots of leguminous plants.

B. Motile; rod-shaped.

C. Mannitol agar brownish; a single polar flagellum.

CC. Mannitol agar fluorescent; four to ten polar flagella.

BB. Non-motile; spherical.

AA. Symbiotic; produce nodules on roots of leguminous plants.

B. Acid in glucose yeast water; serum zone in milk; motile by means of peritrichous flagella.

BB. Alkaline in glucose yeast water; no serum zone in milk; motile by means of single flagellum.

5. *Rhizobium japonicum*.

1. *Azotobacter chroococcum*.

2. *Azotobacter agile*.

3. *Azotobacter vitreum*.

4. *Rhizobium leguminosarum*.

VIII. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE CAPABLE OF OXIDIZING
ALCOHOL TO ACETIC ACID

(The genus *Acetobacter*)

A. Catalase produced.

B. Produce coherent surface growth (film) on beer wort.

C. Bacterial slime produced stains blue with iodine.

CC. Bacterial slime produced does not stain blue with iodine.

BB. Coherent surface growth not formed.

AA. Catalase not produced.

1. *Acetobacter Pasteurianum*.
2. *Acetobacter orleanense*.
3. *Acetobacter Schützenbachii*.
4. *Acetobacter suboxydans*.

**IX. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE PRODUCING
WATER SOLUBLE (green, blue, or yellowish-green) PIGMENTS**

(The genus *Pseudomonas*)

A. Motile.

B. Gelatin liquefied.

C. Nitrates reduced.

D. Litmus milk coagulated and peptonized.

DD. Litmus milk not coagulated.

CC. Nitrates not reduced.

BB. Gelatin not liquefied.

C. Nitrates reduced, and

D. Free nitrogen formed; reddish gray on potato; an odor of trimethylamine not produced.

DD. Nitrites produced; grayish brown on potato; odor of trimethylamine produced.

CC. Nitrates not reduced.

D. Indol formed.

DD. Indol not formed.

1. *Pseudomonas aeruginosa*.

2. *Pseudomonas fluorescens*.

3. *Pseudomonas synchyanea*.

4. *Pseudomonas denitrificans*.

5. *Pseudomonas putida*.

6. *Pseudomonas rugosa*.

7. *Pseudomonas ovalis*.

IX. KEY TO BACTERIACEAE (The genus *Pseudomonas*)—Continued

AA. Non-motile.

B. Gelatin liquefied; indol formed; nitrates reduced; litmus milk peptonized.

8. *Pseudomonas chlorina*.

BB. Gelatin not liquefied; indol not formed; nitrates not reduced; litmus milk unchanged.

9. *Pseudomonas nonliquefaciens*.

**X. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE PRODUCING
WATER INSOLUBLE (red, violet, and yellow) PIGMENTS**

(The genera *Serratia*, *Chromobacterium*, and *Flavobacterium*)

A. Red growth on agar.

B. Gelatin liquefied.

C. Hydrogen produced from glucose; growth poor on urea and without pigment.

1. *Serratia marcescens*.

CC. Hydrogen not produced from glucose; growth vigorous on urea with intense red pigment.

2. *Serratia indica*.

3. *Serratia rosea*.

BB. Gelatin not liquefied.

AA. Growth on agar not red.

B. Violet growth on agar.

C. Indol formed.

CC. Indol not formed.

D. Motile.

DD. Non-motile.

BB. Yellow growth on agar.

C. Gelatin liquefied.

D. Nitrates reduced.

DD. Nitrates not reduced.

4. *Chromobacterium janthinum*.

5. *Chromobacterium violaceum*.

6. *Chromobacterium viscosum*.

7. *Flavobacterium aquatile*.

X. KEY TO BACTERIACEAE (Genus *Flavobacterium*)—Continued

E. Indol not formed.

8. *Flavobacterium suaveolens*.

EE. Indol not formed.

F. Dextrose fermented with acid or acid and gas.

G. Gas produced from dextrose; litmus milk acid, coagulated; peptonized.

9. *Flavobacterium aromaticum*.

GG. Gas not produced (acid only) from dextrose; litmus milk alkaline; peptonized.

10. *Flavobacterium xynzanthum*.

FF. Neither acid nor gas from dextrose.

G. Motile; litmus milk acid.

11. *Flavobacterium acetylicum*.

GG. Non-motile; litmus milk alkaline.

12. *Flavobacterium fecale*.

CC. Gelatin not liquefied.

D. Nitrates reduced to nitrites.

E. Motile.

13. *Flavobacterium denitrificans*.

EE. Non-motile.

14. *Flavobacterium orale*.

DD. Nitrates not reduced.

E. Litmus milk acid and coagulated.

15. *Flavobacterium lactis*.

EE. Litmus milk unchanged.

16. *Flavobacterium butyri*.

XI. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE WHICH ARE GRAM POSITIVE

(The genus *Lactobacillus*)

A. Acid produced from lactose.

B. Acid produced from maltose.

BB. No acid from maltose.

AA. No acid from lactose.

B. Litmus milk acid with soft coagulum.

BB. No change in litmus milk.

1. *Lactobacillus acidophilus*.

2. *Lactobacillus bulgaricus*.

3. *Lactobacillus Beijerinckii*.

4. *Lactobacillus Delbrückii*.

XII. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE PRODUCING GAS FROM LACTOSE

(The genera *Aerobacter*, *Escherichia*, and *Citrobacter*)

A. Produce acetoin (acetyl methyl carbinol) from dextrose.

B. Glycerol and starch fermented with acid and gas; non-motile; gelatin not liquefied.

C. Acid and gas from sucrose.

D. Acid and gas from dulcitol.

DD. Neither acid nor gas from dulcitol.

CC. Neither acid nor gas from sucrose.

BB. Neither acid nor gas from glycerol nor starch; motile; gelatin liquefied (frequently very slowly).

C. Acid and gas from sucrose.

CC. Neither acid nor gas from sucrose.

AA. Do not produce acetoin (acetyl methyl carbinol) from dextrose.

B. Utilize citric acid as sole source of carbon (heavy turbidity in liquid citric acid media).

C. Acid and gas from sucrose.

D. Indol formed; acid and gas from salicin and esculin.

E. Acid and gas from galactose; motile; utilize uric acid.

6. *Citrobacter Freündii*.

EE. Neither acid nor gas from galactose; non-motile; do not utilize uric acid.

7. *Citrobacter diversum*.

DD. Indol not formed; neither acid nor gas from salicin nor esculin.

XII. KEY TO BACTERIACEAE PRODUCING GAS FROM LACTOSE—Continued

- E. Acid and gas from dulcitol.
 EE. Neither acid nor gas from dulcitol.
 CC. Neither acid nor gas from sucrose.
 D. Acid and gas from dulcitol.
 E. Utilize uric acid.
 EE. Uric acid not utilized.
 DD. Neither acid nor gas from dulcitol.
 BB. Do not utilize citric acid as sole source of carbon (turbidity not developed in citric acid medium).
 C. Neither acid nor gas from sucrose.
 D. Acid and gas from salicin.
 DD. Neither acid nor gas from salicin.
 E. Motile.
 EE. Non-motile.
 CC. Acid and gas from sucrose.
 D. Indol formed.
 E. Motile.
 EE. Non-motile.
 F. Acid and gas from salicin.
 FF. Neither acid nor gas from salicin.
 DD. Indol not formed.
8. *Citrobacter anindolicum*.
 9. *Citrobacter sulphidogenes*.
 10. *Citrobacter ovicola*.
 11. *Citrobacter intermedium*.
 12. *Citrobacter glycologenes*.
 13. *Escherichia coli*.
 14. *Escherichia grunthalii*.
 15. *Escherichia acidi-lactici*.
 16. *Escherichia communior*.
 17. *Escherichia neapolitana*.
 18. *Escherichia coscoroba*.
 19. *Escherichia anindolica*.

XIII. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE PRODUCING GAS FROM DEXTROSE BUT NOT FROM LACTOSE

(The genera *Proteus* and *Salmonella*)

- A. Acid and gas from sucrose.
- B. Neither acid nor gas from mannitol.
 - C. Acid and gas from maltose.
 - CC. Neither acid nor gas from maltose.
 - BB. Acid and gas from mannitol.
 - C. Motile; gelatin liquefied.
 - CC. Non-motile; gelatin not liquefied.
- AA. Neither acid nor gas from sucrose.
 - B. Acetoin (acetyl methyl carbinol) produced from dextrose.
 - BB. Acetoin not produced from dextrose.
 - C. Acid and gas from mannitol.
 - D. Acid and gas from maltose.
 - E. Acid and gas from xylose.
 - F. Acid and gas from arabinose.
 - G. Hydrogen sulphide produced.
 - H. Acid and gas from inositol.

- 1. *Proteus vulgaris*.
- 2. *Proteus mirabilis*.
- 3. *Proteus hydrophilus*.
- 4. *Proteus asiaticus*.
- 5. *Salmonella Archibaldii*.

XIII. KEY TO BACTERIACEAE PRODUCING GAS FROM DEXTROSE—Continued

- I. Tartrate media alkaline.
- II. Tartrate media acid.
- HH. Neither acid nor gas from inositol.
 - GG. Hydrogen sulphide not produced.
 - FF. Neither acid nor gas from arabinose.
 - EE. Neither acid nor gas from xylose.
 - DD. Neither acid nor gas from maltose.
 - CC. Neither acid nor gas from mannitol.
- 6. *Salmonella Schotmülleri*.
- 7. *Salmonella aertrycke*.
- 8. *Salmonella enteritidis*.
- 9. *Salmonella abortus-equus*.
- 10. *Salmonella cholerae-suis*.
- 11. *Salmonella paratyphi*.
- 12. *Salmonella pullorum*.
- 13. *Salmonella Morganii*.

XIV. KEY TO SOME SPECIES OF THE FAMILY BACTERIACEAE NOT PRODUCING GAS FROM CARBOHYDRATES

(The genera *Eberthella*, *Shigella*, and *Alcaligenes*)

A. Acid (but no gas) produced from dextrose.

B. Motile.

C. Gelatin not liquefied.

D. No acid from lactose.

E. Acid from mannitol; sucrose not fermented with acid.

1. *Eberthella typhi*.

EE. No acid from mannitol; acid produced from sucrose.

2. *Eberthella talavensis*.

DD. Acid from lactose.

E. Acid from raffinose.

EE. No acid from raffinose.

CC. Gelatin liquefied.

D. Acid from sucrose.

E. Acid from lactose.

EE. No acid from lactose.

DD. Acid not produced from sucrose.

BB. Non-motile.

5. *Eberthella oedematiensis*.

6. *Eberthella enterica*.

7. *Eberthella xenopa*.

3. *Eberthella belfastensis*.

4. *Eberthella pyogenes*.

XIV. KEY TO BACTERIACEAE NOT PRODUCING GAS FROM CARBOHYDRATES—Continued

C. Acid not produced from mannitol.

D. Indol not formed; acid not produced from rhamnose.

8. *Shigella dysenteriae*.

DD. Indol formed; acid produced from rhamnose

9. *Shigella ambigua*.

CC. Acid produced from mannitol.

D. Acid not produced from xylose.

E. No acid from lactose; indol generally formed.

10. *Shigella Flexneri*.

EE. Acid from lactose; indol not formed.

11. *Shigella Sonnei*.

DD. Acid produced from xylose.

E. Acid from lactose and sucrose; litmus milk acid and coagulated.

12. *Shigella dispar*.

EE. No acid from lactose or sucrose; litmus milk alkaline.

13. *Shigella alkalescens*.

AA. Acid not produced from dextrose.

B. Nitrates reduced.

C. Gelatin not liquefied.

CC. Gelatin liquefied.

14. *Alcaligenes faecalis*.

15. *Alcaligenes Bookeri*.

BB. Nitrates not reduced.

C. Gelatin liquefied; milk peptonized.

16. *Alcaligenes Marshali*.

CC. Gelatin not liquefied; milk not peptonized.

17. *Alcaligenes abortus*.

XV. KEY TO SOME GENERA AND SPECIES OF THE FAMILY SPIRILLACEAE

A. Short curved rods; possess one or only two or three polar flagella.

B. Sulphates not reduced to H_2S .

C. Indol formed.

D. Litmus milk not coagulated.

DD. Litmus milk coagulated.

CC. Indol not formed.

D. Gas produced from dextrose; nitrates reduced.

DD. Gas not produced from dextrose; nitrates not reduced.

E. Gelatin liquefied.

F. Milk coagulated.

FF. Milk not coagulated.

EE. Gelatin not liquefied.

BB. Sulphates reduced to H_2S .

AA. Longer spiral rods; possess a tuft of numerous polar flagella.

B. Gelatin not liquefied.

C. Yellow pigment on gelatin, no growth on potato.

CC. Red pigment on potato.

BB. Gelatin liquefied.

1. *Vibrio cholerae*.

2. *Vibrio danubicus*.

3. *Vibrio Leonardii*.

4. *Vibrio proteus*.

5. *Vibrio aquatilis*.

6. *Vibrio percolans*.

7. *Vibrio desulphuricans*.

8. *Spirillum undula*.

9. *Spirillum rubrum*.

10. *Spirillum serpens*.

EXERCISE 67

QUANTITATIVE BACTERIAL ESTIMATION BY DILUTION

The purpose of this exercise is to determine, within certain limits, the number of *Esch. coli* present in one loop of a 24 hour broth culture. The technique described introduces the method employed in the detection and estimation of the incidence of *Esch. coli* as an index of pollution in water.

A. Materials Needed:

1. Twenty-four hour broth culture of *Esch. coli*.
2. Eight tubes of lactose broth in fermentation tubes (Durham tubes preferable).¹
3. Eight tubes of sterile physiological salt solution (9 c.c.).
4. Eight sterile 1 c.c. pipettes in a case.

B. Procedure:

1. Label tubes of broth from one to eight. Similarly label tubes of salt solution from one to eight.
2. Inoculate tube No. 1 of salt solution with one standard 4 mm. loop of the broth culture of *Esch. coli*. Roll and agitate the tube between the palms to distribute the organisms through the solution. Assume that each c.c. of this tube now contains one-tenth the number of bacteria that were present in one loop of the culture. (In practice it would be necessary to have 10 c.c. in this first dilution tube.)
3. With a sterile pipette inoculate tube No. 2 of salt solution with 1 c.c. of the material from tube No. 1. Each c.c. of salt solution tube No. 2 contains 1/100 as many organisms as were present in the original

¹ The Durham fermentation tube consists of a small tube inverted in a larger tube of sugar medium.

loop. (All precautions as to the flaming of plugs and the mouths of tubes must be faithfully carried out.)

With the same pipette inoculate tube No. 1 of broth with 1 c.c. from salt solution tube No. 1.

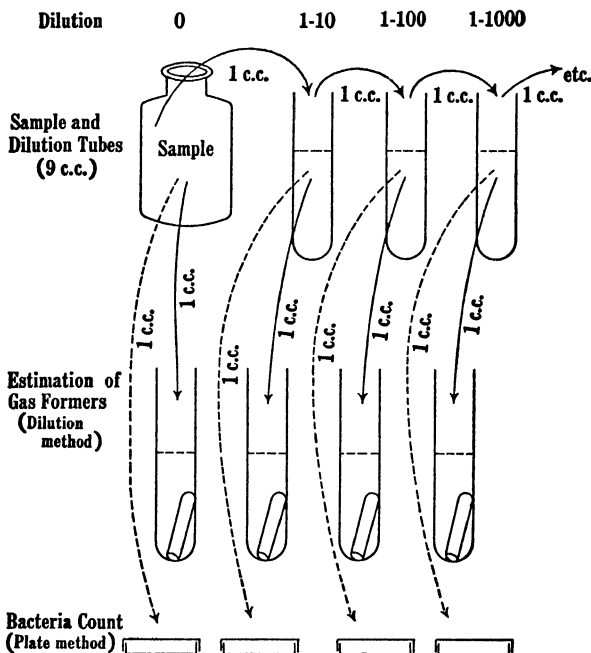


FIG. 9. PROCEDURE FOR ESTIMATION OF BACTERIA BY DILUTION AND PLATING TECHNIQUES.

4. With tube No. 2 of salt solution make transfers into tubes No. 2 of broth and No. 3 of salt solution exactly as indicated above in 3 and 3a. (A new sterile pipette must be used.)
5. Continue the process of inoculation of tubes of broth and tubes of salt solution until all of the broth tubes have been inoculated.

6. Incubate the broth tubes in the 37° thermostat for 24 to 48 hours.

C. Examination and Record:

1. Record for each tube in tabular form whether
 - a. growth occurred.
 - b. gas was formed.
 - c. *Esch. coli* is present.
2. Estimate the number of *Esch. coli* present in one loop of the 24 hour broth culture employed, regarding only those tubes that show gas as positive. All other tubes should show no growth. If growth (without gas) is present it indicates contamination and poor technique and the exercise should be repeated.
3. Assuming that one platinum loop is equal to 1/100 c.c., calculate the number of bacteria per c.c. of the broth culture.

EXERCISE 68

QUANTITATIVE BACTERIAL ESTIMATION BY PLATING

The purpose of this exercise is to afford practice in the technique of plating and to ascertain the number of viable bacteria in a unit volume of a substance. The method employed is fundamental for quantitative bacteriological work.

A. Materials Needed

1. Twenty-four hour broth culture of *Esch. coli*.
2. Eight sterile petri dishes.
3. Eight tubes of agar about one-half full.
4. Sterile pipettes in case.
5. Five tubes physiological salt solution.

B. Procedure:

1. Label tubes of salt solution 1 to 5.
2. Label petri dishes in duplicate numbers 2, 3, 4, and 5, respectively.
3. Add one standard loop of the broth culture of *Esch. coli* to tube No. 1 of physiological salt solution. Assume that each cubic centimeter of this tube now contains one-tenth of the number of organisms present in the loop. (See note in B-2, Exercise 67.) Roll and agitate the tube between the palms to distribute the bacteria evenly.
4. With a sterile one c.c. pipette add one c.c. from the tube No. 1 to tube No. 2. Each cubic centimeter of this tube contains one one-hundredth as many organisms as were present in the original loop.
5. Roll and agitate tube No. 2 between the palms of the hands to distribute the organisms, and then proceed as follows:

- a. With a sterile one c.c. pipette add one c.c. to each of the two petri dishes marked No. 2.
- b. With the same pipette add one c.c. to dilution tube No. 3, thus making a one to one thousand dilution of the loop.
6. Repeat with tube No. 3 as directed for tube No. 2, inoculating tube No. 4 and plates No. 3. Be sure to use a new pipette.
7. Repeat with tube No. 4 as directed for tube No. 2, inoculating tube No. 5 and plates No. 4.
8. Proceed with tube No. 5 as directed for tube No. 2 under 5a above, inoculating plates No. 5.
9. Pour into each petri dish a tube of melted agar which has been cooled to 45° C. Mix the contents, allow to solidify, and incubate in the 37° C. thermostat 24 to 48 hours. Be careful to invert the petri dishes on incubation.

C. Examination and Record:

1. Count the number of colonies on each petri dish and record in tabular form. The product of the number of colonies and the reciprocal of the dilution is the number of bacteria per loop of the original broth culture.
2. Calculate the number of *Esch. coli* per c.c. of a 24 hour broth culture assuming that one loop equals 1/100 of a cubic centimeter.
3. Is the dilution or plating method, in your opinion, more accurate for bacterial enumeration?
4. Does the plate method of counting give a maximum or minimum count? State reasons.

EXERCISE 69

EXAMINATION OF SURFACE WATER

It is the object of this exercise to familiarize the student with the technique and aim of bacterial water analysis.

A. Materials Needed:

1. Four sterile petri dishes.
2. Sterile pipettes in case (1 c.c. and 10 c.c.).
3. One tube of dilution water.
4. Four tubes agar for plates.
5. One large fermentation tube of lactose broth.
6. Two small fermentation tubes of lactose broth.
7. Sample of water from a stream, pond, or lake.

Note: The bacteriological examination of water consists of the determination of:

- (1) The number of bacteria present in a unit volume of water (1.0 c.c.) which are capable of growing on nutrient agar.
- (2) The incidence of intestinal forms, particularly *Esch. coli*.

The bacterial content is determined by plating 1.0 c.c. or smaller portions on agar. The presence of *Esch. coli* is detected by placing measured quantities (10.0 c.c., 1.0 c.c., etc.) in lactose broth fermentation tubes and if gas is formed, *Esch. coli* is regarded as probably present. This is known as the presumptive test for *Esch. coli*. In practice, further tests to ascertain that the gas formed is due to *Esch. coli* are carried out.

B. Procedure:

1. Shake the sample thoroughly to insure even distribution of the organisms, then open the bottle, taking

care not to contaminate the sample with the fingers, etc.

2. Place 10 c.c. of the sample in a large fermentation tube, discard the pipette.
3. Place 1 c.c. of the sample into:
 - a. each of two petri dishes.
 - b. a small fermentation tube.
 - c. a tube of dilution water.Then set aside the pipette.
4. Place 0.1 c.c. of sample (1 c.c. of the diluted sample) into:
 - a. each of two petri dishes.
 - b. a small fermentation tube.
5. Pour into each petri dish a tube of melted agar which has been cooled to 42° to 45° C. Tip the dish back and forth to insure good distribution and put aside to solidify.
6. Place the agar plates in the 37° incubator for 24 hours or until the next period; also incubate the lactose broth fermentation tubes at the body temperature.

C. Examination and Record:

1. Count the number of colonies on each plate and record in tabular form.
2. Record the per cent of gas in the fermentation tubes after 24 and 48 hours. Tabulate.
3. Is the water fit for drinking without purification?
4. What precautions must be taken in the collection of a water sample for bacterial analysis?
5. Calculate the number of bacteria and of gas forming types per c.c. of water.
6. In what respects does the method of analysis here employed differ from standard methods of water analysis?

Ref.: Buchanan. *Bacteriology*, pp. 462-470.

Standard Methods of Water Analysis of A.P.H.A. and A.W.W.A. 1933.

EXERCISE 70

ANALYSIS OF WELL WATER

The procedure in this exercise must be employed if it is desired to ascertain whether the water conforms to the U.S. Treasury Department standard for water on interstate common carriers with respect to gas formers.

A. Materials Needed:

1. Sample of water to be secured by the student in a sterile wide-mouthed bottle.
2. Four tubes of agar two-thirds full.
3. Fermentation tubes filled with lactose broth (5 large, 2 small).
4. Water blanks, one tube.
5. Sterile pipettes and petri dishes.

The sample of water is to be secured by the student from a well in which he is interested. The sample should be secured only after several buckets of water have been pumped out of the well. Use a sterile wide-mouthed bottle for the collection. Make a map of the locality. Obtain the following data in regard to each well:

- a. kind—whether dug, drilled, driven, or bored.
- b. depth.
- c. type of soil.
- d. distance from all buildings, cesspools, etc.
- e. amount of water used daily from the well.
- f. has the well ever run dry?
- g. protection of well against surface washings.

B. Procedure:

1. Shake the sample thoroughly, open carefully, and place five 10 c.c. portions in large fermentation tubes,

also one 1.0 c.c. and one 0.1 c.c. portions into small fermentation tubes.

2. Place 1.0 c.c. in each of two sterile petri dishes, also 0.1 c.c. in each of two petri dishes.
3. Pour a tube of agar (melted and cooled to 42° to 45° C.) into each petri dish.
4. Incubate at 37° C. as in Exercise 69.

C. Examination and Record:

1. Count the number of colonies on each plate and calculate the number of bacteria per c.c.
2. Record gas fermentation tubes after 48 hours.
3. Assuming that the gas is due to *Esch. coli* is the water fit for drinking without purification?¹

See note, Exercise 69.

EXERCISE 71

EXAMINATION OF MILK AND DETECTION OF ACID FORMING BACTERIA

A special method for the detection and isolation of organisms having a distinctive characteristic (acid production) is introduced here.

A. Materials Needed:

1. Sample of milk from boarding place. (Secure in a sterile wide-mouthed bottle.)
2. Two tubes of litmus solution.
3. Six tubes of lactose agar.
4. Six tubes of plain agar (two-thirds full).
5. Four water blanks (nine c.c.).
6. Sterile pipettes and petri dishes.

B. Procedure:

1. Place 1/100, 1/1,000, and 1/10,000 c.c. of the milk into each of four petri dishes. (See Exercise 68.)
2. To two petri dishes of each dilution add a tube of plain agar which has been melted and cooled to 42° to 45° C. Mix thoroughly and set aside to harden.
3. To each of the remaining dishes add one c.c. of sterile litmus and a tube of lactose agar, mix and cool as above.
4. Incubate at 37° C. for 48 hours.

C. Examination and Record:

1. Count and record the number of colonies on each plate of plain agar.
2. Count and record the total and acid colonies on the litmus-lactose-agar.
3. Make Gram stains from four of the acid colonies (select colonies which appear to be different) and re-

cord whether they are Gram positive or negative rods, cocci or spirilla.

4. What is meant by certified milk?
5. What temperature and time is generally employed for pasteurization?
6. Do you consider your sample good milk? Give reasons.

Ref.: Buchanan. *Bacteriology*, pp. 487-493.

EXERCISE 72

THE COLON TYPHOID OR INTESTINAL GROUP OF BACTERIA

This exercise serves to introduce the principle of differentiation of closely related groups of organisms by means of sugar fermentation reactions.

A. Materials Needed:

1. Culture of *Esch. coli*.
2. Culture of *Salmonella cholerae-suis* (or *Sal. pullorum*, *Sal. schotmülleri*, *Sal. para typhi*.)
3. Culture of *Eberthella typhi*.
4. Culture of *Alcaligenes fecalis*.
5. Five tubes of lactose peptone water (with Andrade indicator) in Durham fermentation tubes.
6. Five tubes of glucose peptone water (with Andrade indicator) in Durham fermentation tubes.
7. Five tubes of Russell Double Sugar Agar slants.¹

B. Procedure:

1. Inoculate each culture into one tube of each of the above media. (Inoculate the "Russell Double Sugar" medium by stabbing in the butt and also streaking on the slant.)
2. Incubate at 37° C. for 24 hours (may be left for 48 hours if necessary).

C. Examination and Record:

1. Record in tabular form acid and gas formation in glucose and lactose peptone water.
2. Record in tabular form the appearance of the Russell Double Sugar medium. (Note acid and gas formation in butt and acid production on slant surface.)

¹ Nutrient agar, with 1.0% lactose, 0.1% glucose, and litmus, Andrade or other suitable indicator.

3. Make and record Gram stain from R.D.S. slants.
4. Are any of the organisms studied in this exercise pathogenic? If so, indicate the animals attacked and the disease produced.
5. List four species which are closely related to the species studied in this exercise, and if pathogenic, the disease produced.
6. In what respect does *Esch. coli* differ from the other organisms?

Ref.: Buchanan. *Bacteriology*, pp. 397-413.

EXERCISE 73

DIFFERENTIATION OF INTESTINAL BACTERIA ON SOLID MEDIA

The use of special media for the rapid differentiation of closely related species is illustrated.

A. Materials Needed:

1. Broth culture of *Esch. coli*.
2. Broth culture of *Aero. aerogenes*.
3. Broth culture of *Sal. cholerae-suis* (or other member of the para-typhoid group).
4. Three water blanks (10 c.c.).
5. Three petri dishes containing Endo agar.
6. Three petri dishes containing eosine methylene blue agar.
7. Glass smearing rods (see Ex. 61).

B. Procedure:

1. Transfer a loop of the culture of *Esch. coli* to a tube of dilution water.
2. Place a loop of this diluted culture of *Esch. coli* in the center of a plate of Endo agar, and another on the plate of E.M.B. agar.
3. By means of a glass smearing rod (which should be flamed and cooled just prior to use) distribute the loop of culture over the surface of the agar media.
4. Incubate at 37° C. for 24 or if necessary 48 hours.
5. Proceed with *Aero. aerogenes* and *Sal. cholerae-suis* as directed for *Esch. coli*.

C. Examination and Record:

1. Describe the appearance of the colonies of the various organisms on the two media employed.
2. What is the composition of (a) Endo, and (b) eosine methylene blue agar?

EXERCISE 74

THE NON-SPECIFIC, PYOGENIC COCCI

A. Materials Needed:

1. Pus from pimples, boils, rhinitis, sinusitis, or suppurating wound of an animal.
2. Glucose agar slants.
3. Sterile scalpel and forceps.

B. Procedure:

1. To obtain pus from a pimple or boil, wash the infected area with soap and water, then with alcohol. Open the infection with the scalpel, or pull off the head with the forceps. Obtain some of the pus with a sterile platinum loop or needle, and inoculate a glucose agar slant. Incubate at 37° for 24 to 48 hours.
2. Obtain purulent material from case of rhinitis, etc., in a sterile petri dish or bottle. Inoculate a bit of pus onto a glucose agar slant.
3. Make a Gram stain of a bit of pus. (In making the smear, do not use any diluent, but spread the pus directly on a slide, dry, fix, and stain.)

C. Examination and Record:

1. Examine the mount made directly from the pus and note the prevalence of bacteria, leucocytes, or other materials. Draw. Note the types of bacteria present.
2. Examine the agar slant and describe the growth.
3. Make a Gram stain from the culture and compare it with the direct smear of the pus.

Note: This agar culture, if pure, may be employed for making a vaccine, in Exercise 76.

4. Sketch and label the constituents of pus as observed from the Gram stain. What is the Gram reaction of (a) the bacteria and (b) the leucocytes?
5. List (a) the more common primary infections due to non-specific pyogenic cocci, (b) secondary infections.
6. List three specific pyogenic cocci and the diseases they produce.

Ref.: Buchanan. *Bacteriology*, pp. 370-388.

EXERCISE 75

ANIMAL INOCULATION

(Demonstration)

The function of this exercise is to acquaint the student with the methods commonly employed in animal inoculation, and to impress him with the necessity of using animals.

A. Materials Needed:

1. Sterile hypodermic needles.
2. Five per cent phenol or two per cent lysol.
3. Sterile scissors.
4. Cotton.
5. Rabbits or other animals.

B. Procedure:

Instructor will demonstrate:

1. Subcutaneous injection.
2. Intraperitoneal injection.
3. Intravenous injection.

C. Report:

1. Why are animals essential for bacteriological research?
2. What animals are most frequently employed?
3. List more important methods of inoculation.
4. Describe the procedure of making an intraperitoneal or intravenous injection.

Ref.: Buchanan. *Bacteriology*, pp. 328-329.

EXERCISE 76

PREPARATION OF A VACCINE

In this exercise are introduced the use of the capillary pipette, the technique for preparing blood smears, and another method for counting bacteria.

A. Materials Needed:

1. Agar slant culture of *Esch. coli* or pyogenic coccus (*Staph. aureus*) (24 to 48 hours).
2. Physiological salt solution, four tubes.
3. Citrated salt solution, one tube (2% sodium citrate).
4. Hollow ground slides.
5. Sterile capillary pipettes.
6. Phenol 5%, one tube.
7. Wright's blood stain.

B. Procedure:

1. **Preparation of emulsion.** Place three or four c.c. of the physiological salt solution on the agar culture and emulsify the growth by rolling the tube between the palms of the hands. With some organisms it is necessary to scrape off the growth with a platinum loop before rolling.
2. **Standardization.**
 - a. Place six standard loops of citrated salt solution in the hollow of a hollow ground slide.
 - b. Puncture your finger (or ear) or that of a fellow student and add two standard loops of blood, then two loops of the bacterial emulsion to the citrated salt solution on the slide. Mix thoroughly with the platinum loop.
 - c. Place 2 or 3 loops of the mixture from "b" near one

end of a clean slide (lying flat on the desk) and spread it in a thin film as follows:

- (1) Place the edge of a second slide on the first so as to make an angle of about 45° and pull it towards the drop of blood and bacteria mixture. (When it touches the blood the liquid will run out along its edge.)
- (2) Now push this slide back over the first slide maintaining the angle at 45° .
- d. Stain with Wright's stain, or with Loeffler's methylene blue. (See note at the end of the exercise.)
- e. Count the number of red blood cells and bacteria in at least five fields.
- f. Calculate the number of bacteria per cubic centimeter from the following formula (the number of red cells is taken as 5,000,000 per c.mm.).

$$\text{Bacteria per c.c.} = \frac{5,000,000 \times 1,000 \times \text{No. of bacteria}}{\text{No. of red cells}}$$

3. Sterilization.

- a. Transfer the bacterial emulsion to a clean test tube with a sterile capillary pipette, taking care that the suspension does not flow along the sides of the tube.
- b. Heat the portion of the tube above the emulsion to sterilize it, and when cool place the tube in a water bath at 60° C. for one hour. (The water must be well above the surface of the liquid in the tube. In practice the tube is sealed and entirely immersed in the water bath.)
- c. Transfer two loops of the heated vaccine to an agar slant or broth and incubate for 24 to 48 hours. If growth develops the vaccine cannot be used.

4. Dilution.

- a. Dilute the suspension with physiological salt solution to make 1,000,000,000 organisms per c.c. and add sufficient phenol to make 0.25 to 0.5%.

This suspension is then placed in sterile containers and constitutes the vaccine.

C. Examination and Record :

1. Record whether there was growth on the transfers from the heated suspension.
2. Record name of organism, of individual from whom specimen was taken, bacterial content, date of preparation, and if autogenous vaccine, state the source of the culture.
3. Differentiate between vaccine and bacterin.
4. Define or explain: (a) stock bacterin, (b) autogenous bacterin, (c) univalent bacterin, (d) polyvalent bacterin, (e) mixed bacterin.
5. List two diseases for which bacterins are employed for: (a) prophylactic, and (b) curative purposes.

Note: Method of staining with Wright's stain:

- a. Make films and dry quickly in the air.
- b. Cover the dried film with the stain and allow to act for one minute (to fix). Do not fix by heat.
- c. Add distilled water, drop by drop, until a yellow metallic scum begins to form. Add the drops of water rapidly in order to prevent precipitates on the stained film. Allow to stain for five to ten minutes.
- d. Wash in distilled water until the film has a pinkish tint. Blot dry with filter paper. Red cells are orange to pink; nuclei, various shades of violet.

Ref.: Buchanan. *Bacteriology*, p. 355.

EXERCISE 77

THE MICROSCOPIC (WIDAL) AGGLUTINATION TEST

(Detection of Typhoid Infection)

This exercise introduces the student to a technique for diagnosis of disease by detection of a specific antibody in a patient's serum.

A. Materials Needed:

1. Three hollow ground slides or slides with rings attached.
2. Three large coverslips.
3. Vaseline.
4. Young (24 hour) broth culture of *Eberthella typhi* (or *Salmonella cholerae-suis*).
5. Serum specific for *Eberthella typhi* (or *Salmonella cholerae-suis*).
6. Normal serum (of rabbit).
7. Physiological salt solution.

B. Procedure:

1. Make a hanging drop (as was done in the examination for motility) using one small loop of physiological salt solution and one small loop of the broth culture of *Eberth. typhi* (or *Sal. cholerae-suis*). (This is a control.)
2. Make another hanging drop, using one loop of the diluted (1 to 20 or 1 to 50) normal serum and one loop of the broth culture of *Eberth. typhi* (or *Sal. cholerae-suis*).
3. Similarly make a hanging drop with a loop of diluted immune serum (1 to 20 or 1 to 50) and a loop of the broth culture.
4. Let stand 30 minutes to one hour and examine with the high power dry lens.

C. Examination and Record:

1. Draw, showing appearance of the three slides. If agglutination takes place the organisms lose their motility and form characteristic masses or clumps.
2. Define: antigen, antibody, agglutinin, precipitin.
3. What is the agglutination test frequently used for?
4. What was the object of using the salt solution and normal serum controls?
5. Describe briefly how you would proceed to ascertain whether a man had typhoid fever by the agglutination test.

Ref.: Buchanan. *Bacteriology*, pp. 343-345.

EXERCISE 78

THE MACROSCOPIC AGGLUTINATION TEST

A. Materials Needed:

1. Young (24 to 48 hour) agar slant culture of *Eberth. typhi* (or *Sal. cholerae-suis*). (Growth should be all over the surface.)
2. Agglutinating serum for *Eberth. typhi* (or *Sal. cholerae-suis*), two c.c. of 1 to 40 dilution in a tube.
3. Agglutination tubes (10).
4. Sterile capillary pipettes.
5. Physiological salt solution (three tubes).
6. Rack for agglutination tubes.

B. Procedure:

1. Place the tube containing two c.c. of 1 to 40 dilution of serum in the rack and label No. 1.
2. Label nine other tubes from Nos. 2 to 10, respectively, put them in the rack, and place in each of them one c.c. of physiological salt solution.
3. Label the remaining tube C (control) and put into it one c.c. of physiological salt solution.
4. With a sterile pipette remove one c.c. from tube No. 1 to tube No. 2, mix and then add one c.c. from tube No. 2 to tube No. 3, etc., until tube No. 10 is reached, then discard one c.c. from tube No. 10. This will give a series of dilutions of serum ranging from 1-40 to 1-20, 480.
5. To the agar culture of *Eberth. typhi* (or *Sal. cholerae-suis*) add two or three c.c. of salt solution. Roll the tube gently between the palms of the hands. The organisms should form a heavy even suspension.¹
6. With the capillary pipette add one or two drops of the

¹ A suspension of killed organisms may be employed in place of the above if desired.

MACROSCOPIC AGGLUTINATION TEST 179

bacterial suspension to each tube of serum and to the control and mix by gently tapping the tubes. (The suspension should show a distinct but not a heavy turbidity.)

7. Incubate at 37° C. for two hours and record whether agglutination has occurred.
8. Leave in the locker and record again the next morning.

C. Examination and Record:

1. Record presence or absence of agglutination after two hours' incubation and again after standing at room temperature over night.

Note: Agglutination is manifested as follows: The even turbidity changes to a granular appearance and eventually the agglutinated bacilli fall to the bottom leaving a clear supernatant liquid.

2. Which do you consider more accurate, the microscopic or macroscopic agglutination tests? Why?

SUPPLEMENTARY EXERCISES
IN
SANITARY AND FOOD
BACTERIOLOGY

Directions in the following exercises are purposely somewhat less detailed, though, where feasible, the general form employed in the fundamental exercises is adhered to. It is anticipated that the references given will be consulted before, or in conjunction with, performance of the experiments.

EXERCISE 79

MICROORGANISMS RESPONSIBLE FOR TASTES AND ODORS IN WATER

The object of the following exercise is to acquaint the student with some types of microorganisms, other than bacteria, which have been responsible for tastes, odors, or other objectionable conditions in water supplies. It is not the purpose to study the life histories of the organisms but primarily to afford an opportunity to become acquainted with the appearance, and a few of the salient differential characteristics of these undesirable water forms.

A. Materials Needed:

1. Clean microscope slides.
2. Clean cover glasses.
3. Platinum loop.
4. Clean pipettes (preferably with large openings).
5. Fresh or preserved specimens of:
 - a. Diatoms.
 - b. Cyanophyceae (blue green algae).
 - c. Schizomycetes (not including bacteria).
 - d. Chlorophyceae (green algae).
 - e. Protozoa, and other animal forms.

B. Procedure:

Examine each of the materials furnished as follows:

1. Place a small amount on a microscope slide: add a drop of water and cover with a clean coverslip.
2. Examine with the low power of the microscope (first surveying at least a dozen fields), then draw the various types of organisms present.

Note: (Whipple's *Microscopy of Drinking Water* or other suitable guide should be available in the laboratory for ready reference.)

3. Re-examine the specimen using the high power dry lens and make drawings.
4. Identify as many forms as possible.

Ref.: Whipple, G. C. (Revised by Fair, G. M., and Whipple, M. C.). 1927. *The Microscopy of Drinking Water*. John Wiley and Sons. New York.

Ward, H. B., and Whipple, G. C. 1918. *Fresh Water Biology*. John Wiley and Sons. New York.

West, G. S. 1916. *Algae. I. Myxophyceae, Peridinieae, Bacillarieae, Chlorophyceae, Together with a Brief Summary of the Occurrence and Distribution of Fresh Water Algae*. University Press. Cambridge, England.

EXERCISE 80

MICROSCOPIC ORGANISMS IN POOLS, LAKES, MUD, RESERVOIRS, ETC.

A. Materials Needed

1. Microscope slides, cover glasses, pipettes.
2. Samples of:
 - a. Scums from stagnant pools, watering troughs, lakes, reservoirs, etc.
 - b. Mud from river, lake, reservoir bottoms, or dead ends of water distributing systems.

B. Procedure:

1. Examine as described in Exercise 79 and identify as many of the forms as possible.
2. Sketch and label the microscopic organisms observed.

Ref.: Purdy, W. C. 1922. A Study of the Pollution and Natural Purification of the Ohio River. Part I. Plankton and Related Organisms. U.S. Public Health Bulletin, No. 131.

Kofoed, C. A. 1923. "Microscopic Organisms in Reservoirs." *Jour. A.W.W.A.*, 10, 183.

EXERCISE 81

QUANTITATIVE MICROSCOPIC ANALYSIS OF WATER

(The Sedgwick-Rafter Method)

A. Materials Needed:

1. Whipple ocular micrometer.
2. Stage micrometer.
3. Cylindrical funnel (Sedgwick-Rafter) for filtration.
4. Counting cell (brass or rubber ring, 1 mm. high cemented to microscope slide).
5. Coverslips.
6. Bolting cloth.
7. Berkshire sand or quartz.
8. Small beaker or large test tubes.
9. Wide-mouthed pipettes.

B. Procedure:

1. Standardization of Whipple eye piece.

- a. Place the Whipple ocular micrometer in the eye piece of the microscope.
- b. Adjust the draw tube of the microscope so that a side of the ocular micrometer exactly covers 1 mm. on the stage micrometer. (Use the 16 mm. objective.)
- c. Record the length of the draw tube for future reference.
- d. Familiarize yourself with the divisions on the ocular micrometer. Note particularly the smallest square which is 1 "Standard Unit," and that the total area of the entire square on the ocular micrometer is 1 sq. mm.

Note: By enumerating the organisms present in 1 such field (using the Whipple counting cell,

which is 1 mm. in thickness), the total number of organisms per cubic mm. is determined.

2. Filtration of the sample.

- a. Place a small amount of sand into the neck of the filter funnel, using sufficient sand to give a filtering depth of $1/2$ inch.
- b. Moisten a piece of bolting cloth. Place it on the rubber stopper of the cylindrical filter and insert it into the neck of the funnel. The sand is thus supported upon this perforated rubber stopper and prevented from falling through by the bolting cloth.
- c. Place the funnel in an upright position and add cautiously a few c.c. of the water to be filtered so as to wet the sand but not disturb it any more than is necessary. Then slowly add the remaining (500 to 1000 c.c.) water to be filtered. Allow filtration to continue until 5 c.c. is left in the funnel as indicated by the graduations.

3. Separation of the organisms from the sand.

- a. Remove the stopper and catch the sand and the 5 c.c. of water above it in a clean beaker or test tube.
- b. Shake the beaker gently to disengage organisms from the sand and decant quickly to a second test tube or beaker. This will effect a separation of the particles of sand from the organisms.
- c. Wash the sand in the first beaker or tube with 5 c.c. of water and decant into the second as before.

Note: The effect of filtration and washing has been to concentrate the organisms present in the original sample. The degree of concentration is expressed by the ratio of the volume of the sample filtered to the volume of water employed in washing. For example: If 1000 c.c. were filtered down to 5 c.c., and

then 5 more c.c. were used to wash the sand, the total volume of the water containing the organisms would be 10 c.c. and there would be a concentration of 100 times.

4. Examinations and enumeration.

- a. Shake up the contents gently to distribute the organisms evenly throughout the liquid and fill the counting cell by means of a wide-mouthed pipette.
- b. Place a clean coverslip on the counting cell, taking care to exclude air bubbles.
- c. Examine with the low power of the microscope and count 10 fields. (Use Whipple eye piece.) Record the number of each kind of organism present and the approximate area covered in terms of standard units for each field, on the chart furnished. (It is essential to examine through the entire depth of the counting cell.)

5. Calculation of the microscopic content of the sample.

- a. *The Sedgwick-Rafter factor.* The Sedgwick-Rafter factor is that number by which the total number of organisms counted must be multiplied in order to reduce the results to the number of organisms present per cubic centimeter of the original sample. It is expressed by the formula:

$$F = \frac{1000 W}{nf}$$

when W = the number of c.c. of water used in washing, n = the number of squares or fields counted, and f = total volume in c.c. of water filtered. The total number of organisms per unit volume (1 c.c.) of the original sample is equal to X times F , where X is the total number of organisms counted and F is the Sedgwick-Rafter factor.

QUANTITATIVE MICROSCOPIC ANALYSIS 189

6. Records.

Record as follows:

Sample No. Source.

Date collected..... Examined.....

Volume water filtered. . . . c.c. Flotation

Concentrated to..... c.c. Factor.....

[illegible]

- a. Define the term "Standard Unit."
- b. What are the important errors in the method?
- c. Describe briefly some other method of microscopic analysis (collection of samples, etc.), and indicate its advantages and disadvantages as compared with the Sedgwick-Rafter method.

Ref.: Fair and Whipple. *Microscopy of Drinking Water*, pp. 71-133.
Standard Methods of Water Analysis. 1933. pp. 99-110.

EXERCISE 82

RELATION OF MEDIUM AND TEMPERATURE OF INCUBATION TO BACTERIAL COUNT

(Gelatin at 20° C., and Agar at 20° C., and 37° C.)

A. Materials Needed:

1. Samples of water from the following sources:
Well, cistern, pond, creek or river, sewage.
2. Sterile pipettes.
3. Sterile dilution water (9 c.c. in test tubes and 99 c.c. in bottles).
4. Sterile petri dishes.
5. Gelatin and agar in bottles, flasks, or tubes.

B. Procedure:

1. Make appropriate dilutions and plate out in duplicate from each dilution.
(For waters of fairly good quality, such as wells and reservoirs, dilutions of 1 to 10 and 1 c.c. are desirable. For rivers and cisterns 1 to 10, 1 to 100, and 1 to 1000, and for sewage 1 to 100, 1 to 1000, and 1 to 10,000 or more, may be necessary. It is the object to dilute so as to have from 50 to 400 colonies develop on a plate.) There will be six plates poured from each dilution (2 with gelatin and 4 with agar).
2. Incubate the gelatin and 2 of the agar plates from each dilution at 20° C. The remaining 2 agar plates from each dilution will be incubated at 37° C.

C. Examination and Record:

1. Count the 20° C. plates after 48 hours and those incubated at 37° C. after 24 hours
2. Record and compare counts.

Ref.: Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 45-54. Wiley and Sons. New York.

EXERCISE 83

DIRECT ISOLATION OF ORGANISMS OF THE COLON GROUP BY PLATING ON SOLID MEDIA

A. Materials Needed:

1. Samples of polluted waters and sewage.
2. Sterile petri dishes.
3. Sterile pipettes.
4. Litmus solution.
5. Lactose agar in tubes or bottles.
6. Fuchsin sulphite (Endo) agar in tubes or bottles.
7. Bill salt (MacConkey) agar in tubes or bottles.
8. Agar slants.
9. Lactose broth in Durham fermentation tubes.

B. Procedure:

1. Place 1 c.c. of various dilution of the sample into each of 3 petri dishes.
2. To 1 petri dish add 1 c.c. of litmus and lactose agar, to the second, add fuchsin sulphite agar, and to the third add MacConkey agar.
3. Incubate at 37° C. for 24 hours

C. Examination and Record:

1. Record the total number and the number of acid colonies developing on each medium.
2. Assuming that all the red colonies are *Esch. coli*, which of the three media seems to be most useful for isolation of *Esch. coli* by direct plating of water?
3. Fish 1 colony from each medium on to an agar slant and incubate at 37° C.
4. Make a Gram stain from the agar slant culture; if the organism is found to be a Gram negative, short rod,

without endospores, inoculate into lactose broth, incubate at 37° C., for 24 to 48 hours and record if gas is formed.

5. Characterize the colon group of bacteria.

Ref.: Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 55-92.

Levine, Max. 1921. Bacteria Fermenting Lactose and Their Significance in Water Analysis. Bull. 62, Iowa Engineering Experiment Station, pp. 43-48.

Standard Methods of Water Analysis. 1933. pp. 166-168.

EXERCISE 84

PRESUMPTIVE TESTS FOR THE COLON GROUP IN WATER

(Preliminary Enrichment)

A. Materials Needed:

1. Sample of a polluted water.
2. Dextrose broth in Smith fermentation tubes and in Durham fermentation tubes.
3. Lactose broth in Durham fermentation tubes.
4. MacConkey's bile salt neutral red medium in Durham fermentation tubes.
5. Lactose bile in Durham fermentation tubes.
6. Gentian violet lactose broth in Durham fermentation tubes.
7. Brilliant green lactose bile in Durham fermentation tubes.
8. Sterile pipettes.
9. Dilution water.
10. Broth cultures of *Esch. coli*, *Aero. cloacae*, *Aero. aerogenes*, *B. acetoehtylicum*, and *Cl. welchii* or other lactose fermenting anaerobe.

B. Procedure:

1. Place 1 c.c. of the following dilutions of the water provided into fermentation tubes of each medium designated above. Dilution 1-10, 1-100, 1-1000, 1-10,000. Place the dextrose broth in Durham fermentation tubes in 46° C. incubator (after warming to that temperature) if available. All others are stored in 37° incubator for 24 to 48 hours.
2. Place a loop of *Esch. coli* into a fermentation tube of each medium and incubate as above.
3. Repeat with each of the cultures as with *Esch. coli*.

C. Examination and Record:

1. Record the amount of gas in each tube in each medium after 24 and 48 hours.
2. Determine the gas ratio in dextrose broth in Smith fermentation tube using the highest dilution that shows gas. Record whether there is a characteristic fluorescens in the MacConkey bile salt medium.
3. Save the tube of each medium of highest dilution which shows gas for use in the following exercise.
4. Criticize the gas ratio as determined in the Smith tube.
5. Indicate briefly the purposes and principle underlying the presumptive tests.
6. Discuss briefly the relative value of the various media tested as presumptive tests.

Ref.: Standard Methods of Water Analysis. 1933. pp. 124-126.

Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 61-78.

Levine, Max. 1921. Bacteria Fermenting Lactose and Their Significance in Water Analysis. Bull. 62, Iowa Eng. Expt. Sta., pp. 48-60.

Creel, R. H. 1914. Examination of Drinking Water on Railway Trains. Bull. 160, Hyg. Lab. U.S.P.H.S. Washington.

Levine, Max. 1918. "Presumptive Tests for *B. Coli*." *Jour. Am. Water Works Assn.*, **5**, 168-171.

———. 1920. "Experiences with the Presumptive and Confirmatory Tests for *B. coli* in Water Analysis for the American Expeditionary Forces." *Jour. Am. Water Works Assn.*, **7**, 188-192.

Jordon, H. E. 1932. "Brilliant Green Bile for *Coli aerogenes* Group Determination." *Jour. Am. Water Works Assn.*, **24**, 1027.

EXERCISE 85

ISOLATION OF COLON GROUP AFTER PRELIMINARY ENRICHMENT

(Partially Confirmed Test for Colon Group)

A. Materials Needed:

1. Positive presumptive test tubes (preferably 24 and not over 48 hours old).
2. Sterile petri dishes.
3. Litmus lactose agar in petri dishes or in tubes.
4. Endo agar in petri dishes or in tubes.
5. Eosine methylene blue agar in petri dishes.
6. Conradi-Drigalski agar in petri dishes.
7. Bent glass rods.

B. Procedure

Isolation may be accomplished in the following ways:

Method 1. Barely touch the liquid in the preliminary enrichment tube, showing gas, with the point of a platinum needle, wash off this needle in a tube of melted litmus lactose or Endo agar, and pour into a petri dish. Incubate at the body temperature for 24 hours.

Method 2. Pour L.L.A., Endo, or other differential agar media into petri dishes and allow to solidify and dry in the incubator. Dip a very small loop into the positive preliminary enrichment tube and wash it off in a tube of sterile salt solution. Place a loop of this wash water in the center of a plate containing the differential medium and spread it over the surface with the aid of a sterile glass rod bent at right angles. Incubate 20 to 24 hours at body temperature.

Method 3. Dip a platinum needle, whose end is bent at an angle of about 120° , into the positive preliminary enrichment tube, stab into a plate containing the differential medium (to remove the excess organisms) and then make a series of streaks on this plate about a quarter of an inch apart, taking care to always streak in the same direction and to lift the needle at the end of each stroke. With a little practice, very excellently isolated colonies may be obtained by this method. Incubation is, as before, at body temperature for 24 hours.

1. Select four positive presumptive test tubes and isolate organisms, using each method given above.
2. Repeat with cultures of *Esch. coli* using each method.

C. Examination and Record:

1. Record presence of colonies resembling *Esch. coli* colonies.
2. Fish one suspicious colony from each medium, and one colony known to be *Esch. coli* onto agar slants for use in the following exercise.

Ref.: Levine, Max. 1921. Bull. 62, Iowa Eng. Expt. Sta., pp. 46-48.

EXERCISE 86

CONFIRMATORY TESTS FOR COLON GROUP OF BACTERIA

A. Materials Needed:

1. Cultures of suspected colon forms fished from previous exercise, and *Aerobacter aerogenes*, *Aerobacter cloacae*, and *Escherichia coli*.
2. Lactose broth.
3. Gelatin.
4. Litmus milk.
5. Tryptophane broth.
6. Kovac's indol reagent.

B. Procedure:

1. Inoculate each of the organisms fished and the pure cultures into each of the media designated above.
2. Incubate gelatin at 20° C., and all other media at 37° C. for 48 hours.

C. Examination and Record:

1. Make Gram stain of agar slant cultures. Record the Gram reaction and see if spores are present.
2. **Lactose broth.** Record for each organism gas formation and acid production in lactose broth.
3. **Litmus milk.** Record acid production; decoloration of litmus; coagulation; digestion.
4. **Tryptophane broth.** Has indol been produced?
5. **Gelatin.** Was gelatin liquefied? (Leave for two weeks if necessary.)

Ref.: Levine, Max. 1921. Bull. No. 62, Iowa Eng. Expt. Sta., pp. 60-64.

Standard Methods of Water Analysis. 1933. pp. 126-130.

EXERCISE 87

DIFFERENTIATION OF MEMBERS OF THE COLON GROUP ON SOLID MEDIA

(Cultural Characters)

A. Materials Needed:

1. Nutrient agar slants.
2. Petri dishes containing Endo agar.
3. Petri dishes containing eosine methylene blue agar.
4. Cultures of broth, preferably lactose broth.
 - a. *Escherichia coli*.
 - b. *Escherichia communior*.
 - c. *Escherichia acidi-lactici*.
 - d. *Aerobacter aerogenes*.
 - e. *Aerobacter cloacae*.
 - f. *Aerobacter levans*.
 - g. *Citrobacter sulphidogenes*, or *Citro. intermedium*, or *Citro. ovicola*.

B. Procedure:

1. Inoculate each of the cultures furnished onto nutrient agar slants.
2. Inoculate each of the cultures furnished onto Endo and eosine methylene blue agar as described for *Method 2* in Exercise 85.
3. Incubate at 37° C. for 20 to 24 hours.

C. Examination and Record:

1. Compare the growths on the agar slants, as to vigor, elevation, consistency, odor, and light transmission.
2. Describe the appearance of the colonies of the various cultures on Endo and E.M.B.

3. Reincubate the plates for another 24 or 48 hours and describe any changes in colony characteristics.

Ref.: Levine, Max. 1921. Bull. 62, Iowa Eng. Expt. Sta., pp. 61-64.

Harris, N. MacL. 1925. "The Preparation of Endo's Medium." *The Military Surgeon*, **57**, 280.

Levine, Max. 1918. "Differentiation of *B. coli* and *B. aerogenes* on a Simplified Eosine Methylene Blue Agar." *Jour. Infect. Dis.*, **23**, 43-47.

EXERCISE 88

DIFFERENTIATION AND CLASSIFICATION OF THE COLON GROUP ON THE BASIS OF BIOCHEMICAL REACTIONS

A. Materials Needed:

1. Cultures (preferably agar slants) of
 - a. *Escherichia coli*.
 - b. *Escherichia communior*.
 - c. *Escherichia acidi lactici*.
 - d. *Aerobacter aerogenes*.
 - e. *Aerobacter cloacae*.
 - f. *Aerobacter levans*.
 - g. *Citrobacter sulphidogenes*, or *Citro. intermedium*, or *Citro. ovicola*.
2. Durham fermentation tubes containing the following carbon compounds in broth or peptone water (0.5% peptone, 0.2% K_2HPO_4 , 0.5% test substance, and 1% Andrade's indicator).
 - a. Sucrose.
 - b. Dulcitol.
 - c. Glycerol.
 - d. Starch.
 - e. Cellobiose.
3. Nutrient gelatin.
4. Citric acid medium (1.5 g. sodium-ammonium-phosphate [micro-cosmic salt]; 1 g. potassium-dihydrogen-phosphate; 0.001% phenol red indicator; 0.2 g. magnesium sulphate; 3 g. sodium citrate [crystals]; per liter distilled water).
5. Clark and Lubs medium.
6. Tryptophane broth.
7. Proteose iron citrate agar (for H_2S detection).
8. O'Meara's reagent (0.3% creatin in 40% NaOH).
9. Methyl red indicator (0.1% alcoholic solution).

B. Procedure:

1. Inoculate each of the organisms furnished into each of the test media (gelatin and proteose iron citrate agar by stabbing).

2. Incubate at 37° C. for 24 to 72 hours (the Clark and Lubs medium should preferably be incubated for 5 days at 30° C., if suitable incubator is available, and gelatin must be kept at 20–23° C.).

C. Examination and Record:

1. Record:

- a. Acid and gas production in media in fermentation tubes.
- b. Growth and change in reaction in citric acid.
- c. Indol production in tryptophane broth.
- d. Hydrogen sulphide production in proteose iron citrate agar.
- e. Gelatin liquefaction (this medium may have to be stored two weeks).
- f. Reaction to methyl red and presence of acetyl methyl carbinol in the Clark and Lubs medium as follows:
 - (1) To a portion of the medium add a few drops of methyl red and note reaction (acid or alkaline).
 - (2) To the remainder of the test medium add an equal volume of 40% NaOH containing 0.3% creatin and let stand exposed to the air. Development of an eosine pink coloration in 15 minutes to 24 hours constitutes a positive Voges-Proskauer test, i.e., for acetyl methyl carbinol.

Note: The V.P. test may be performed after 24 hours at 30° C. or 37° C. The M.R. test should be performed preferably after 5 days at 30° C.; (for class purposes 3 to 4 days at 37° C. is generally satisfactory).

2. Tabulate the results and correlate with colony appearance as found in Exercise 87.
3. Make a dichotomous key for the organisms studied.
4. Indicate briefly possible differences, if any, in sanitary

significance of presence of the various organisms in a water.

Ref.: Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 55-58.

MacConkey, A. 1905. "Lactose Fermenting Bacteria in Feces." *Jour. Hyg.*, **5**, 33. 1909.

———. 1909. *Jour. Hyg.*, **9**, 86.

Kligler, I. J. 1914. "Studies on the Classification of the Colon Group." *Jour. Infect. Dis.*, **15**, 187.

Levine, Max. 1918. "A Statistical Classification of the Colono-cloacae Group." *Jour. Bact.*, **3**, 253-276.

———. 1921. Bull. 62, Iowa Eng. Expt. Sta., pp. 32-42.

EXERCISE 89

BACTERIOLOGICAL EXAMINATION OF WATER

A. Materials Needed:

1. Samples of water from various sources; tap, reservoir, cistern, lake, stream, etc.
2. Lactose broth (30-40 c.c. in large Durham fermentation tubes to be used for 10 c.c. samples).
3. Lactose broth (7 to 10 c.c. in small Durham fermentation tubes, to be used for 1 c.c. or smaller samples of water).
4. Nutrient agar in tubes or bottles.
5. Eosine methylene blue or Endo agar, in petri dishes.
6. Nutrient agar slants.

B. Procedure:

1. Place five-10 c.c., one 1 c.c. and one 0.1 c.c. of the sample into lactose broth and incubate at 37° C.
2. Place 1 c.c., 0.1 c.c. (or smaller quantities if considered necessary) into each of 4 petri dishes, add nutrient agar, and incubate two plates of each quantity of sample at 37° C. (24 hrs.) and the remaining plates at 20-22° C. (48 hrs.).

C. Examination and Record:

1. After 24 hours incubation
 - a. count the nutrient agar plates incubated at 37° C., record, and discard.
 - b. record, quantity of gas present in lactose broth tubes.

If there is any evidence of gas, plate out from the tubes showing gas onto eosine methylene blue or Endo agar as described in Ex. 85 and incubate at 37° C. for 20 to 24 hours, and then

- (1) record appearance of colonies (coli-like, aerogenes-like, questionable, etc.).
- (2) fish suspicious colonies into lactose broth and onto nutrient agar to ascertain whether the colonies are Gram negative non-sporing lactose fermenters.

If the tubes do not show gas replace them in the 37° C. incubator for an additional 24 hours (48 hours total incubation) and treat those tubes which develop gas as described in "b" above.

2. After 48 hours incubation count the nutrient agar plates stored at 20-22° C.
3. Tabulate results.
4. Which of the steps carried out constitute
 - a. the presumptive test,
 - b. the partially confirmed test,
 - c. the completely confirmed test as defined in Standard Methods of Water Analysis?
5. In what respects does the above procedure differ, if any, from Standard Methods?
6. Compare the bacterial analysis of the samples with the U.S. Treasury Department Standard for drinking water.

Ref.: Standard Methods of Water Analysis. 1933. pp. 136-154.

EXERCISE 90

DETECTION AND ESTIMATION OF SPORE FORMING LACTOSE-FERMENTING BACTERIA

(Relation to Presumptive Test)

A. Materials Needed :

1. Samples of soil, sewage, manure, etc.
2. Durham fermentation tubes containing:
 - a. dextrose broth.
 - b. lactose broth.
 - c. lactose bile.
 - d. gentian violet lactose broth.
 - e. brilliant green lactose bile.
3. Sterile pipettes.
4. Sterile dilution water.

B. Procedure :

1. Heat a suspension of soil, manure, or sewage sample in a water bath at 80° C. for 10–15 minutes, taking care that the liquid in the bath is above that of the test sample, or bring the test sample to a boil.
2. Inoculate various quantities of the heated test material into the different sugar media and incubate at 37° C., 24 to 72 hours.

C. Examination and Record :

1. Record gas, or acid and gas production after 24 and 48 hours (or longer if desired).
2. If gas is produced proceed as described in Exercise 85, Method 2, and record results.
3. What is the significance of this experiment from the

standpoint of interpretation of the presumptive test for the colon group? Explain.

Ref.: Levine, Max. 1921. Bull. 62, Iowa Eng. Expt. Sta., pp. 91-98.

Hinman, J. J., and Levine, Max. 1922. "A Facultative Spore-forming Lactose-fermenting Organism from Iowa Surface Waters." *Jour. Am. Water Works Assn.*, **9**, 330.

Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 121-124.

EXERCISE 91

DETECTION OF SEWAGE STREPTOCOCCI

A. Materials Needed:

1. Dextrose broth in Durham fermentation tubes.
2. Sewage or feces suspension.
3. Litmus lactose agar in tubes.
4. Eosine methylene blue agar in petri dishes.

B. Procedure:

1. Inoculate various dilutions of the sewage or feces suspension into dextrose broth. Incubate at 37° C.
2. After 24 hours or less (preferably as soon as there is any evidence of gas) proceed as follows:
 - a. Make Gram stain of the fermenting material.
 - b. Plate out onto litmus lactose agar (as in Method 1, Ex. 85), and onto eosine methylene blue agar (as in Methods 2 or 3, Ex. 85). Incubate at 37° C.
3. Replace dextrose-broth tubes in 37° C. incubator for 2 or 3 days, then repeat 3a, and 3b above.

C. Examination and Record:

1. Record observations on Gram stains of dextrose broth (12 to 24 hour and 72 to 96 hour cultures).
2. Examine the litmus lactose agar plates for presence of small brick red colonies and record Gram stains from such colonies as well as others.
3. Record characters of colonies on eosine methylene blue agar; Gram stains, etc.
4. Discuss the possible effect of presence of streptococci on the Standard Methods of Water Analysis procedure for detection and isolation of members of the colon group.

Ref.: Prescott and Winslow. 1924. *Elements of Water Bacteriology*, pp. 114-121.

EXERCISE 92

BACTERIOLOGICAL EXAMINATION OF OYSTERS REMOVED FROM THE SHELL

(Opened or Shucked Stock)

A. Materials Needed :

1. Lactose broth fermentation tubes.
2. Containers with 200 c.c. of a 2% salt solution graduated to 400 c.c.
3. Dilution tubes containing 9 c.c. of 1% NaCl.
4. Sterile pipettes.

B. Procedure :

1. To 200 c.c. of sterile 2% salt solution in a suitable container add oyster meats sufficient to bring the water level up to the 400 c.c. graduation mark. Shake thoroughly. The watery fluid is used for the determination of incidence of *Escherichia coli* and closely related forms.
2. Place five 1 c.c. portions of the diluted sample, and five 1 c.c. portions of 1 to 10 and 1 to 100 dilutions of the liquor into fermentation tubes containing standard lactose broth.
3. Confirm according to Standard Methods of Water Analysis. From the fermentation tubes showing gas transfers are made on Endo or E.M.B. agar plates; suspicious colonies are then transferred to a second lactose fermentation tube and to a slant agar tube. Make a microscopic examination of the agar slant. If desired, determination of the type of the colon organism may be made on the basis of the V.P. and M.R. reactions, etc. (See Exs. 87 and 88.)

C. Record:

1. The results of the bacteriological examination for the colon group are expressed by the following arbitrary numerical system, known as the American Public Health Association Method of Scoring Oysters.
2. If desired, higher dilutions may be run. In this case a 1 to 1000 dilution has a positive value of 1000 and a 1 to 10,000 dilution of 10,000 and so on.
3. The presence of the colon group in each fermentation tube, if confirmed, is to be given the following values, which represent the reciprocals of the greatest dilutions in which the test for the colon group is positive:

If present in 1.0 c.c. but not in 0.1 c.c. the value is 1.

If present in 0.1 c.c. but not in 0.01 c.c. the value is 10.

If present in 0.01 c.c. the value is 100.

The addition of these values for the five fermentation tubes gives the total value for the sample and this figure is the score, the tube representing the greatest dilution for each set being counted. The results are expressed in the following tabular form:

RESULTS OF TESTS FOR COLON GROUP IN DILUTIONS INDICATED

FERMENTATION TUBES	1.0 c.c.	0.1 c.c.	0.01 c.c.	NUMERICAL VALUE
1	+	+	0	10
2	+	+	0	10
3	+	0	0	1
4	+	0	0	1
5	+	0	0	1

Score 23

+ Positive; confirmed colon group in fermentation tube.

0 Negative; colon group absent.

4. Sometimes results similar to the following are obtained; that is, one or more tubes may show positive results in small quantities of test sample while other

tubes may show negative results in larger quantities. In this case a recession of values is made and the next lower numerical value is given to the positive results in the higher dilution and such positive result is considered as being transferred to a lower dilution giving a negative result in another set of tubes.

As examples of the method of obtaining the score, the following table is given:

RESULTS OF TESTS FOR COLON GROUP IN DILUTIONS INDICATED

TUBES	1.0 c.c.	0.1 c.c.	0.01 c.c.	VALUE
1	+	+	0	10
2	+	+	0	10
3	+	+	0	10
4	+	0	0	10
5	+	+	+	10
Score 50				
1	+	+	0	10
2	+	+	0	10
3	+	+	+	100
4	+	+	+	10
5	+	0	0	10
Score 140				

5. In what respects does this examination differ from that recommended for shucked oysters? Oysters in the shell?

Ref.: Prescott and Winslow. *Elements of Water Bacteriology*, Chapter X.

Tanner, F. W. 1932. *Microbiology of Food*, pp. 433-451.

Hunter and Harrison. 1928. U.S.D.A. Bull. No. 64.

Bundesen. 1925. *Jour. Am. Med. Assoc.*, **84**, 841-850.

Krumwiede. 1926. *Am. Jour. Pub. Health*, **16**, 142-152.

Tooney and White. 1926. *Am. Jour. Pub. Health*, **16**, 597-602.

Wells. 1929. *Am. Jour. Pub. Health*, **19**, 72-79.

1922. *Am. Jour. Pub. Health*, **12**, 574-576.

EXERCISE 93

PHENOL COEFFICIENT

(Preliminary or Practice Experiment)

The method used in this experiment simulates that employed for determination of the "Hygienic Laboratory Phenol Coefficient." (Modifications are made for convenience of laboratory exercise.)

A. Materials Needed:

1. Broth cultures (24 hour) of *Esch. coli*.
2. Sterile funnel (small) with sterile filter paper.
3. Sterile test tubes, 6 (short, wide tubes most suitable).
4. Pipette, 1 c.c. graduated in tenths (sterile).
5. Three (or more) platinum loops, 4 mm. in diameter.
6. Two sterile 100 c.c. graduates.
7. Two 5 c.c. delivery pipettes, sterile.
8. Six sterile bottles.
9. Phenol.
10. Two flasks containing each about 300 c.c. sterile distilled water.
11. Wooden blocks. (Enough to give 7 rows of 6 holes each.)
12. Thirty tubes plain broth, 10 c.c. in each. (Lactose broth may be used for preliminary or practice test.) (Broth made by the method adopted by the American Public Health Association.) Reaction pH 7.0.

B. Procedure:

1. **Dilutions of phenol.** The phenol should first be melted by placing the bottle in a pan of warm water. Measure 95 c.c. of sterile distilled water in a sterile graduate and add to it 5 c.c. of phenol using a sterile 5 c.c. pipette. This makes the 5% stock dilution. Using the second sterile graduate make up the following dilutions from this stock dilution beginning with the most dilute.

DILUTION	STOCK 5% PHENOL	DISTILLED WATER
1:70	10 c.c.	25 c.c.
1:80	10 c.c.	30 c.c.
1:90	10 c.c.	35 c.c.
1:100	10 c.c.	40 c.c.
1:110	10 c.c.	45 c.c.

Place each dilution in a carefully labeled bottle. (Other methods for preparing the desired dilutions may be employed.)

- 2. Organism.** Shake the culture of *Esch. coli*, and filter through sterile filter paper into a sterile test tube. (For actual standard determination *Eberthella typhi* must be employed.)
- 3. Seeding tubes.** Place five sterile test tubes in a row in a wooden block and mark each one with the dilution of phenol it is to contain. Starting with the lowest dilution (i.e., the strongest) shake the bottle, and measure 5 c.c. into the tube marked to receive that strength, using a 5 c.c. delivery pipette. Blow out as much of the remaining liquid as possible, then fill the pipette with the next dilution and discard that, then fill the pipette a second time and empty into the second seeding tube. Proceed in this manner with each dilution.
- 4. Subculture tubes.** Arrange the tubes of broth in wooden racks in six rows of five tubes each directly back of the five seeding tubes and subculture tubes. Flame the cotton plugs.
- 5. Method of conducting the test.** The culture is first added to the seeding tubes containing various dilutions of disinfectants in quantities of 1/10 c.c. to each tube. To add the culture, the seeding tube containing the disinfectant is removed from the block with the left hand, slanted at an angle of 45°, and with the right hand the end of the pipette containing the

culture is introduced and lightly touched to the side of the tube where the liquid has run away on account of the slanting. At the proper time the culture is allowed to run into the disinfectant solution, the pipette removed, the tube straightened up, gently shaken a few times, and replaced in the block. Thirty seconds later the second tube is likewise inoculated and so on until all of the seeding tubes have been inoculated. (It will be found helpful to have students work in groups and the timekeeper give a "Ready" signal three or four seconds in advance and "Go" at the moment for inoculation.)

Thirty seconds after the last seeding tube has been inoculated, a transfer of a loop of material should be made from the first seeding tube to the first subculture tube. (Sterile platinum loops, 4 mm. in diameter and bent at an angle of about 135° are used in making these transfers. The loop is inserted into the seeding tube (without removing the latter from the rack) until it touches the bottom, withdrawn, and the plant made into the broth. Each subculture tube is thus inoculated at intervals of thirty seconds, every effort being made to insert and withdraw the loops in a uniform manner until, at the end of fifteen minutes every subculture tube has been planted. After each loop has been used it is handed to an assistant who returns a cooled sterile loop, if a special apparatus for supplying sterile cooled loops is not available.

The tubes in each rack should be marked plainly with dilution of test material, time of exposure, and any other necessary details.

6. **Incubation.** Subculture tubes should be incubated at 37° C. for 48 hours.

C. Examination and Record:

Record growth in the subculture tubes in tabular form, as in the following example:

SAMPLE	DILUTION	TIME CULTURE EXPOSED TO ACTION OF DISINFECTANT IN MINUTES					
		21/2	5	71/2	10	121/2	15
Phenol	1:70
	1:80	+
	1:90	+
	1:100	+	+	+
	1:110	+	+	+	+	+	..

1. In what respects does the method described above differ from:
 - a. the standard Hygienic Laboratory procedure.
 - b. the Rideal-Walker method.
 - c. the Department of Agriculture (F.D.A.) method of testing disinfectants.

Ref.: Anderson, J. F., and McClintie, T. B. 1912. Standardization of Disinfectants. Hygienic Laboratory Bulletin, No. 82. U.S. Public Health Service.

Disinfectant Testing by Hygienic Laboratory Method. Reprint No. 675. U.S. Public Health Service, 1921, or Public Health Reports, 36, 1559-1564.

EXERCISE 94

STANDARDIZATION OF DISINFECTANTS BY F.D.A. METHOD

(Slightly Modified)

A. Materials Needed:

1. Culture of *Eberthella typhi* (Hopkins strain), *Staphylococcus aureus* (F.D.A. strain), 22-26 hours broth culture 37° C., transferred daily, 4 days.
2. Sterile tubes (25 mm. diameter) for disinfecting mixture, 10 or more.
3. Nutrient broth (see note) 30 tubes.
4. Platinum loops (4 mm. diameter, 23 gauge wire).
5. Sterile distilled water.
6. Sterile dilution flasks.
7. Sterile 5 or 10 c.c. pipettes graduated in 0.1 c.c.
8. Phenol. 1-60 and 1-70 for *Staph. aureus* or 1-90 and 1-100 for *Eberthella typhi*.
9. Various dilutions of test disinfectant.

B. Procedure:

1. Place 5 c.c. of the various dilutions of phenol and test disinfectant into the 25 mm. seeding tubes, and label Nos. 1-10.
2. Place 0.5 c.c. of the broth culture of the test organism into tube No. 1; shake to distribute the organisms, and at 30 second intervals thereafter add 0.5 c.c. of culture to the remaining tubes containing the various dilutions of disinfectants. When tube No. 10 is inoculated, 4½ minutes will have elapsed. 30 seconds later proceed as follows:
3. Transfer one standard loop from tube No. 1 to a tube of broth, and at 30 second intervals thereafter transfer a loop from each successive disinfecting mixture

to a fresh tube of broth until three transfers have been made from each of the disinfecting mixtures. This will give three series of inoculations into broth after 5, 10, and 15 minutes' exposure of the test organisms to the various concentrations of disinfectants.

4. Incubate broth tubes at 37° C. for 48 hours.

C. Examination and Record:

1. Record in tabular form presence or absence of growth.
2. Compute F.D.A. phenol coefficient. (Ratio of highest dilution of test disinfectant to highest dilution of phenol which kills the test organism in 10 minutes but not in 5 minutes.)

Notes:

(a) Medium for subcultures.

Liebig's beef extract.....	5 grams
Sodium chloride, c.p.....	5 grams
Armour's peptone.....	10 grams
Distilled water	1000 c.c.

Adjust reaction to pH 6.8 with sodium hydroxide.

Filter through paper, distribute in 10 c.c. portions in tubes, and sterilize at 15 pounds for 40 minutes.

(b) Test culture.

Keep stock cultures on agar medium as above (solidified with 1½ per cent agar-agar) and adjusted to pH 7.2 to 7.4.

For use, transfer culture to the broth medium daily for at least 4 days but not more than one month.

The broth culture is shaken for 15 minutes, allowed to settle 10 minutes and the supernatant liquid transferred to a sterile tube

with a capillary pipette before use. It should be within the following ranges of resistance.

ORG.	CONC. PHENOL	GROWTH AFTER		
		5 min.	10 min.	15 min.
<i>Eberth. typhi</i>	1-90	+	+	-
	1-100	+	+	+
" *	1-90	-	-	-
	1-100	+	+	-
" *	1-90	+	-	-
	1-100	+	+	+
<i>Staph. aureus</i> *	1-60	+	-	-
	1-70	+	+	+

* Preferable combinations.

Ref.: Ruehle, G. L. A., and Brewer, C. M. 1931. U.S. Food and Drug Administration Methods of Testing Antiseptics and Disinfectants Circular No. 198. U.S.D.A.

Shippen, L. P. 1928. "A Fallacy in Standard Method of Examination of Disinfectants." *Am. Jour. Pub. Health*, **18**, 1231-1234.

U.S.P.H.S. 1921. Disinfectant Testing by Hygienic Laboratory Method. Reprint No. 675 or Public Health Reports, **36**, 1559-1564.

Reddish, G. F. 1927. "Examination of Disinfectants." *Am. Jour. of Pub. Health*, **17**, 320-329.

Brewer, C. M., and Reddish, G. F. 1929. "Comparison of Hygienic Laboratory with Department of Agriculture Method for Testing Disinfectants." *Jour. Bact.*, **17**, 44.

EXERCISE 95

MICROANALYSIS OF TOMATO PRODUCTS

(Howard's Revised Method)

A. Materials Needed:

1. Microscope.
2. Howard cell.
3. Stage micrometer (for microscope adjustment).
4. Graduated cylinder.
5. Erlenmeyer flask.
6. Beaker.
7. Thoma-Zeiss counting cell.

B. Procedure and Record:

1. Standardize the microscope so as to give magnifications of 90, 180, and 500 diameters and examine the counting cell to become thoroughly familiar with its rulings.
2. **Molds.** Clean the Howard cell so that the Newton's rings are produced between the slide and the cover glass. Remove the cover and place, by means of a knife blade or scalpel, a small drop of the sample on the central disk; spread the drop evenly over the disk and cover with the cover glass so as to give an even spread to the material. It is of the utmost importance that the drop be mixed thoroughly and spread evenly; otherwise the insoluble matter and the molds are most abundant at the center of the drop. Squeezing out of the more liquid portions around the margin must be avoided. In a satisfactory mount, Newton's rings should be apparent when finally mounted and none of the liquid should be drawn across the moat and under the cover glass.

Place the slide under the microscope and examine with a magnification of about 90 diameters and with such adjustment that each field of view represents approximately 1.5 sq. mm. of area on the mount.¹

Observe each field as to the presence or absence of mold filaments and note the result as positive or negative. Examine at least 50 fields, prepared from two or more mounts. No field should be considered positive unless the aggregate length of the filaments present exceeds approximately one-sixth of the diameter of the field. Calculate the proportion of positive fields from the results of the examination of all the observed fields and report as percentage of fields containing mold filaments.

3. **Yeasts and spores.** Fill a graduated cylinder with water to the 20 c.c. mark, and then add the sample till the level of the mixture reaches the 30 c.c. mark. Close the graduate, or pour the contents into an Erlenmeyer flask, and shake the mixture vigorously fifteen to twenty seconds. To facilitate thorough mixing the material should not fill more than three-fourths of the container in which the shaking is performed. For tomato sauce or pastes or products running very high in the number of organisms, or of heavy consistency, 80 c.c. of water should be used with 10 c.c. or 10 g. of the sample. In the case of exceptionally thick or dry pastes it may be necessary to make an even greater dilution.

Pour the mixture into a beaker. Thoroughly clean the Thoma-Zeiss counting cell so as to give good Newton's rings. Stir thoroughly the contents of the beaker with a scalpel or knife blade, and then, after allow-

¹ Comment by Howard and Stephenson: In order to have an area of 1.5 sq. mm. the diameter of the microscopic field should be 1.382 mm. This is determined by using a stage micrometer and adjusting the length of the microscope draw tube. Obviously after the proper draw tube length has been secured the adjustment should be noted and always used in making the mold counts.

ing to stand three to five seconds, remove a small drop and place upon the central disk of the Thoma-Zeiss counting cell and cover immediately with the cover glass, observing the same precautions in mounting the sample as given above. Allow the slide to stand not less than ten minutes before beginning to make the count. Make the count with a magnification of about 180. (Approximately with 8 mm. objective and $\times 10$ eye piece.)

Count the number of yeasts and spores¹ on one-half of the ruled squares on the disk (this amounts to counting the number in eight of the blocks, each of which contains twenty-five of the small ruled squares). The total number thus obtained equals the number of organisms in 1/60 c.mm. if a dilution of one part of the sample with two parts of water is used. If a dilution of one part of the sample with eight parts of water is used the number must be multiplied by three. In making the counts the analyst should avoid counting an organism twice when it rests on a boundary line between two adjacent squares.

4. **Bacteria.** Estimate the bacteria from the mounted sample used above, but allow the sample to stand not less than fifteen minutes after mounting before counting.² Employ a magnification of about 500 (8 mm. objective, $\times 20$ eye piece, draw tube extended to about 190).

Count and record the number of bacteria in a small area consisting of five of the small-sized squares. Move the slide to another portion of the field and count the number on another similar area. Count five such areas, preferably one from near each corner of the ruled portion of the slide and one from near the cen-

¹ Comment by Howard and Stephenson: The organisms counted as "yeasts and spores" are the yeast cells and yeast and mold spores, not bacteria spores.

² The estimation of yeast, molds, and bacteria are made from the same mount.

ter. Determine the average number of bacteria per area and multiply by 2,400,000 which gives the number of bacteria per cubic centimeter. If a dilution of one part of the sample with eight parts of water (instead of two parts of water) is used in making up the sample, then the total count obtained as above must be multiplied by 7,200,000. Omit the micrococci type of bacteria in making the count.

5. **Yeast and spore count.** The ruled square on the slide is 1 mm. on each side and the cell is $1/10$ mm. deep. The volume of the ruled part is, therefore, $1/10$ c.mm. The ruled area is divided into sixteen large squares and the number of organisms is counted in eight of these, which is equivalent to $1/2$ of $1/10$ c.mm. or $1/20$ c.mm. If a dilution of one part of the product to two parts of water is used, $1/3$ of $1/20$ c.mm., or $1/60$ c.mm. as representing the actual amount of original stock in which organisms are counted, is obtained.
6. **Bacterial count.** The average number of bacteria counted on five areas, each consisting of five small squares, multiplied by 2.4 million, equals the number of bacteria per cubic centimeter. In calculating the bacteria, it is observed that there are 400 (20×20) small squares on the slide. The number of bacteria in several areas, each containing five of these small squares, are counted and an average made. This average represents the bacteria in $1/80$ of the total ruled area. Since the cell is $1/10$ mm. deep, the volume represented by the organisms counted is $1/80 \times 1/10$ or $1/800$ c.mm. With the usual dilution of one part of product to two parts of water the actual volume in which the number of organisms is determined is $1/3$ of $1/800$ cm. or $1/2400$ c.mm. or $1/2,400,000$ c.c.
7. In laboratory report on the examination of tomato

products, discuss the value, accuracy, and function of the Howard method of analysis, indicate any possible improvements which suggest themselves to you and describe method of manufacture of tomato products with special reference to possibilities of contamination.

Ref.: Bigelow, W. D. 1920. "Report on Canned Vegetables." *Jour. Assoc. Offc. Agr. Chemists*, **3**, 453.

Darling. 1922. Tech. Bull. No. 91, N.Y. Agri. Expt. Sta.

Howard, and Stephenson. 1917. Bull. 581, U.S. Dept. Agri.

Tanner, F. W. 1932. *Microbiology of Foods*.

EXERCISE 96

INFLUENCE OF COMPOSITION OF FOOD, AND NATURE OF CONTAMINATION, ON DE- COMPOSITION PRODUCTS

A. Materials Needed:

1. Four flasks or bottles each containing 50 c.c. of the following medium:
Water 1000 c.c.
Peptone 1 g.
Gelatin 20 g.
Reaction pH 7.0 to 7.4.
2. Four flasks or bottles each containing 50 c.c. of the above medium plus 1.0% lactose.
3. Culture of *Esch. coli*.
4. Culture of *B. subtilis* or *Ps. fluorescens (liquefaciens)* or *Flavobacterium suaveolens* or some other proteolytic organism.
5. A 50% formalin solution, neutral to phenolphthalein.
6. Phenolphthalein indicator.
7. Burette.
8. N/10 or N/20 NaOH.
9. Evaporating dish or clean small E. flask.
10. Five and 10 c.c. pipettes (clean but not necessarily sterile).
11. Freshly boiled distilled water neutral to phenolphthalein.
12. H-ion indicator solutions.

B. Procedure:

1. Mark four bottles containing gelatin, A, B, C, and D and the four containing lactose with gelatin AL, BL, CL, and DL.
2. Inoculate A and AL with *Esch. coli*.
3. Inoculate B and BL with one of the proteolytic bacteria.
4. Inoculate C and CL with both *Esch. coli* and the proteolytic organisms employed in 3.
5. Incubate in locker for about 5 days including bottles D and DL as controls.

C. Examination and Record:

1. Determine the total acidity, approximate pH, and ammonia and amino nitrogen (by formol titration) for each bottle. Tabulate the results and draw conclusions as to influence of composition of food and nature of contamination on type of spoilage.

Note: The formol titration may be carried out as follows:

- a. To distilled water which has been boiled and cooled add 1.0% phenolphthalein indicator and neutralize to a faint pink.
- b. Dilute formalin with an equal volume of water, add 1.0% phenolphthalein indicator and neutralize.
- c. Place 5.0 c.c. of the medium into an evaporating dish or E. flask.
- d. Add 10 c.c. of the neutralized distilled water ("a") above.
- e. Titrate to neutrality. This gives the total acidity (express in terms of % normality).
- f. Add 10 c.c. of the neutral formaldehyde ("b" to "e") above.
- g. Again titrate to neutrality. This is a measure of the NH_3 and amino nitrogen content.

(To convert to ammonia and amino nitrogen employ the following factors): 1.0 c.c. N/10 NaOH = 1.7 mg. ammonia (NH_3) or 1.4 mg. N. Express results either as mg. N. or ammonia per liter of medium.

2. Illustrate by chemical equations the reactions involved in the formol titration.
3. Give an explanation which you consider feasible as to the influence of carbohydrate or proteolysis in the presence of pure and mixed cultures.

Ref.: Levine and Soppeland. Proteolysis by Bacteria from Creamery Wastes. 1926. Bull. 82, Iowa Eng. Expt. Sta.

EXERCISE 97

BACTERIA IN CANNED FRUITS AND VEGETABLES

A. Materials Needed:

1. Can opener.
2. Mercuric chloride solution, 1-500.
3. Alcohol.
4. Sterile 1 c.c. pipettes.
5. Sterile petri dishes.
6. Plain agar and glucose brom-cresol-purple agar in bottles.
7. Malt extract agar.
8. Dilution water (9 c.c. and 99 c.c.).
9. Gelatin tubes.
10. Glucose agar tubes (at least 1/2 full).
11. Clean slides.
12. Meat tubes.

B. Procedure and Record:

1. Record the appearance of the exterior of the can, whether swelled, leaking, rusty, etc.
2. Wash the top of the can with soap and water.
3. Pour a small amount of alcohol on the top of the can and burn off.
4. With a sterile can opener, open the can.
5. Record the appearance and odor of the material. (*Do not taste.*)
6. Make plate counts in duplicate on plain agar. Incubate 48 hours to 1 week at 37° C. and another series at 55-60° C. Record number of bacteria and acid formers per gram of food.
7. Repeat above, using malt extract agar, incubate at room temperature, and record yeasts and mold colonies after 3 to 7 days.

8. Inoculate various quantities into gelatin tubes and record presence of gelatin liquefiers (incubation, 1 week at 20° C.).
9. Make deep glucose agar shake cultures and inoculate meat tubes. Incubate at 37° C. and 55° C. for 3 days to 1 week. Record presence of anaerobes.
10. Make a direct smear of the food, stain by Gram's method, and record types of organisms present.
11. If a duplicate can is available, incubate can at 55° C., for 5–10 days, open, and test according to paragraphs 5, 6, and 10, but incubate at the higher temperature only. If the can should swell or otherwise indicate bacterial growth in less than 5 days, test immediately.
12. In what respects would the procedure be changed for various products, such as fruits, meat, and vegetables?

Ref.: Tanner, F. W. 1932. *Microbiology of Food*, pp. 554–564.

EXERCISE 98

BACTERIA IN WHOLE EGGS

A. Materials Needed :

1. Soap.
2. Brush or cloth.
3. Mercuric chloride (1-500) or phenol (2%).
4. Alcohol.
5. Tripod or ring stand.
6. Sterile triangle.
7. Wide-mouthed flask containing 100 c.c. broth and some pieces of broken glass or beads.
8. Flask containing 100 c.c. broth.
9. Case sterile pipettes, 1 c.c. graduated in 1/10 c.c.
10. Scales.
11. Scalpel and forceps.
12. Petri dishes, 10.
13. Agar. 10 tubes or 1 bottle.
14. Bunsen burner.
15. Fresh and stored eggs.

B. Procedure :

1. Wash egg in soap and water (with brush or clean cloth).
2. Immerse in 1-500 mercuric chloride or 2% phenol for 1-15 min.
3. Wash off disinfectant with alcohol.
4. Place egg on sterile triangle on tripod and burn off alcohol.
5. Make opening (about 1/2 in. diameter) in acute pole of egg with a sterile scalpel and forceps, taking every precaution against contamination. Replace on sterile triangle pointed end down.
6. Expel white into a weighed flask containing about 100 c.c. broth and some broken glass. This may be done by gently heating the flat end of the egg.

7. Similarly expel the yolk into a weighed flask containing 100 c.c. broth.
8. Weigh each flask.

C. Examination and Record:

1. Shake thoroughly flask containing egg white and place 1 c.c. of the mixture into each of two petri dishes.
2. Similarly place 1/10 c.c. into each of two petri dishes.
3. Treat the yolk sample as above.
4. Add about 10 c.c. melted agar (cooled to 42–45° C.) to each dish.
5. Incubate flasks and petri dishes 2 to 5 days at 20° C.
6. Count plates and record.
7. Calculate the number of bacteria per gram of egg.
(Bact. per gram of egg=no. of bact. per c.c. of broth-egg mixture multiplied by $\frac{100+\text{weight of egg}}{\text{weight of egg}}$)
8. Make Gram and carbol fuchsin stains from incubated flasks of white and yolk. Sketch the types of organisms present, if any.
9. Compare the above procedure with those of (a) Bushnell and Mauer, (b) Rettger, (c) Hadley and Caldwell.

Ref.: Tanner, F. W. 1932. *Microbiology of Food*, pp. 518–524.

Bushnell and Mauer. 1914. Bull. 201, Kansas Ag. Exp. Sta.

Hadley and Caldwell. 1916. Bull. 164, Rhode Island Ag. Exp. Sta.

Rettger. 1913. Bull. 75, Conn. Ag. Exp. Sta.

EXERCISE 99

GROWTH OF BACTERIA IN EGG WHITE

A. Materials Needed:

1. Soap.
2. Brush or cloth.
3. Mercuric chloride (1-500) or phenol (2%).
4. Alcohol.
5. Sterile triangle.
6. Scalpel and forceps.
7. Bunsen burner.
8. Inoculating needle.
9. Sterile (wide mouth) flasks of 150 to 250 ml. capacity (empty).
10. Cultures of several of the following: *Serratia marcescens*, *Serratia rubrica*, *Ps. flavescens*, *Ps. fluorescens*, *Ps. mucidolens*, *Ps. graveolens*, and *B. ichthogenes*.
Any other cultures of special interest may also be used.
11. Strictly fresh eggs.

B. Procedure:

1. Prepare the egg and open, using the same technique as given under Exercise 98.
2. Expel the white into the sterile flask as directed in Exercise 98.
3. Inoculate the egg white with the culture selected for study.
4. Reserve one flask for control.
5. Incubate at 20° C. to 30° C. for 1 to 2 weeks.

C. Examination and Record:

Record any changes in consistency, odor, color, and transparency.

EXERCISE 100

BACTERIAL CHANGES IN EGGS

A. Materials Needed:

1. Fresh eggs.
2. 1-500 HgCl₂ solution.
3. Alcohol.
4. Triangle.
5. Sterile flasks or petri dishes.
6. Scalpel.
7. Sealing wax.
8. Cultures of organisms: *Esch. coli*, *B. subtilis*, *Ps. pyocyaneus*, *Ps. fluorescens*, *Serr. marcescens*, *Clostr. sporogenes*, *Ps. mucidolens*, and *Ps. graveolens*.

B. Procedure:

1. Wash and sterilize two or more eggs as in exercise on bacteria in whole eggs.
2. Separate the white and yolk of one into two sterile flasks or petri dishes as previously instructed.
3. Chip a small hole in the small end of the second.
4. Inoculate the white and the yolk of one egg and the interior of another egg with one of the organisms.
5. Seal the opening in the second egg with sealing wax.
6. Incubate at 20-30° C. for a week.

C. Examination and Record:

1. Record appearance of white and yolk in flasks.
2. Open the sealed egg and pour contents into a petri dish. Describe appearance.

Ref.: Levine, Max, and Anderson, D. Q. 1932. "Two New Species of Bacteria Causing Mustiness in Eggs." *Jour. of Bact.*, **23**, 337-347.

EXERCISE 101

BACTERIA IN FROZEN EGGS

A. Materials Needed:

1. Can of frozen eggs.
2. Alcohol.
3. Drill (sterilized).
4. Spoon (sterile).
5. Sterile container for samples (mason jars are very satisfactory).
6. Flasks with known quantities (45 c.c.) physiological salt solution (sterile).
7. Sterile wide-mouthed pipettes or tubing.

B. Procedure:

1. Wash the top and edge of the cover with a 1-500 solution of HgCl_2 . Pour on a little alcohol around edge of cover, burn off and remove cover.
2. With a sterile drill (sterilize by wetting with alcohol and burning off) bore holes in the frozen eggs as follows: (a) near the center of the can, (b) near the edge of can, and (c) midway between these two points.
3. Collect the shavings with the aid of a sterile spoon in a sterile wide-mouthed glass stoppered bottle or mason jar.
4. As quickly as possible after thawing, stir sample thoroughly (with the aid of an electric stirrer, such as a malted milk stirrer, if possible).
5. Remove about 5 c.c. with a pipette or tube to a weighed flask containing a known volume of physiological salt solution.
6. Weigh flask with sample to 0.1 g. (Increase in weight is weight of sample.)
7. Plate out on nutrient agar. (If desired, lactose broth tubes may be inoculated with the various dilutions.)

8. Incubate the plates for 5 days at 20° C. and count. (If lactose broth tubes were employed determine the presence of the colon group as described in Exercises 85-88.)
9. Calculate and record the number of bacteria per gram.

Note: Bact. per gram = Plate count

$$\times \frac{\text{wt. of sample} + \text{dil. water}}{\text{wt. of sample}}$$

Ref.: Redfield, H. W. 1920. Examination of Frozen Egg Products and Interpretation of Results. U.S.D.A. Chem. Bull. 846.

EXERCISE 102

BACTERIAL CONTENT OF HAMBURGER STEAK OR SAUSAGE

A. Materials Needed:

1. Sterile paper (pieces about 4 or 5 inches square).
2. Sterile mortar and pestle.
3. Sterile clean sand.
4. Flask containing 90 c.c. of sterile water.
5. Five tubes 9 c.c. water blanks.
6. Sterile 1 c.c. pipettes.
7. Sterile petri dishes.
8. Nutrient agar in bottles.
9. Nutrient gelatin.
10. Endo agar in bottles.

B. Procedure:

1. Weigh out 10 grams of commercial hamburger on sterile paper, transfer to a sterile mortar, add a small quantity of sterile sand from time to time, and grind until the meat is fine. Transfer to a flask containing 90 c.c. of sterile water. Shake vigorously 5 minutes. This gives practically a 1-10 dilution.

Note: If sausage is used remove casing with sterile scalpel or forceps then proceed as for hamburger.

2. From this dilution make further dilutions of 1-100, 1-1000, 1-10,000, 1-100,000, and 1-1,000,000.
3. Plate in duplicate on plain agar from each dilution beginning with the 1-100. Incubate at room temperature for 7 days. (For convenience of laboratory schedule it may be necessary to incubate at 37° C., for 1 to 2 days.)

4. Plate in duplicate on Endo agar from each dilution beginning with the 1-100. These plates are for direct enumeration of organisms of the colon group. Incubate for 24 hours at 37° C.
5. Inoculate 1 c.c. from each dilution, including the 1-10, into a tube of melted gelatin. Cool to solidify and incubate for 7 days at about 20° C.

C. Examination and Record :

1. Count the total number of colonies developing on the nutrient agar and record the number of organisms per gram of meat.
2. Count the number of red colonies developing on the Endo agar and record the number per gram of meat. Confirm two red colonies for members of the colon group. (See Exs. 85-88.)
3. Test the tubes of gelatin for liquefaction by cooling in ice. Calculate the approximate number of liquefiers per gram of meat from the results. What is the special significance of this group of organisms in meat?

Ref.: Hoffstadt, R. E. 1924. *Am. Jour. Hyg.*, **4**, 33-51.

Weinzirl and Newton. 1915. *Am. Jour. Pub. Health*, **5**, 833-835.

EXERCISE 103

DETECTION OF SPORE FORMING AN-AEROBES IN SOIL AND FOODS

A. Materials Needed:

1. Dextrose agar tubes.
2. Sterile deep petri dishes.
3. Beef heart medium or meat medium in tubes.
4. Brain medium tubes.
5. Litmus milk (preferably in potato tubes with marble seal).
6. Dextrose broth (preferably in potato tubes with marble seal).
7. Hollow ground slide.
8. Cultures of *Clostridium sporogenes* or *Cl. welchii* or other anaerobe, garden soil, spoiled canned foods of various types.

B. Procedure and Record:

1. Make dextrose agar shake cultures (various dilutions), of each organism, a soil infusion which has been heated at 80° C. 10 to 20 minutes, or the spoiled canned food, and incubate at 37° C. until colonies are formed (2 to 4 days).
2. Examine and draw colonies under low power of microscope.
3. Fish 2 separate colonies and inoculate them into beef heart medium.

Note: Expel the cylinder of media into a sterile petri dish and dissect away until desired colony is obtained.

4. After several days' incubation, make cultures from the beef heart into deep brain, litmus milk, and dextrose broth. Note changes in media in 1 day, 2 days, and 2 weeks.

5. Make Gram stains from the brain and milk cultures (lower portions).
6. Make hanging drops from the dextrose broth to test for motility.
7. Make spore stains from the beef heart or brain (lower portions).
8. Draw several organisms, sporulating and non-sporulating.

EXERCISE 104

SEPARATION OF SPORE FORMING ANAEROBES FROM SPORE FORMING AEROBES

A. Materials Needed:

1. Nutrient agar slants.
2. Beef heart or brain medium.
3. Gentian violet dextrose broth in potato tubes with marble seal (1:100,000 or less of gentian violet in dextrose broth).
4. Mixture of *Clostridium sporogenes* and *Bacillus subtilis*.
5. Capillary pipettes.

B. Procedure:

1. Make cultures from the mixtures as follows:
 - a. Plain aerobic agar slant.
 - b. Beef heart or brain medium.
 - c. Gentian violet dextrose broth with marble seal.
2. Incubate at 37° C. until growth appears in gentian violet medium. If none occurs in 2-3 days reinoculate.
3. Inoculate a second series of the above media (1a, 1b, and 1c) from gentian violet dextrose broth culture which has shown growth below marble seal, after the liquid above the marble seal of the gentian violet medium has been removed with aid of a capillary pipette.
4. Repeat incubation and reinoculation until aerobic culture has been eliminated, as indicated by growth in gentian violet and heart or brain medium coupled with lack of growth on nutrient agar slant.

C. Record:

Record number of trials for successful separation. Discuss theory of separation.

Ref.: Manual of Methods. 1932. Society of American Bacteriologists. Leaflet III.

Hall, I. C. 1920. "Practical Methods in the Purification of Obligate Anaerobes." *Jour. of Infect. Dis.*, **27**, 576.

EXERCISE 105

SOME DIFFERENTIAL CHARACTERISTICS OF FOOD POISONING AND CLOSELY RELATED BACTERIA

A. Materials Needed:

1. Cultures of the following organisms: *Esch. coli*, *Aero. aerogenes*, *Sal. Morgani*, *Sal. paratyphi*, *Sal. Schulmülleri*, *Sal. enteritidis*, *Shigella dysenteriae*, *Shigella Flexneri*, *Eberth. typhi*.
2. Glucose broth with Andrade indicator.¹
3. Lactose broth with Andrade indicator.¹
4. Sucrose broth with Andrade indicator.¹
5. Inositol broth with Andrade indicator.¹
6. Mannitol broth with Andrade indicator.¹
7. Xylose broth with Andrade indicator.¹
8. Litmus milk.
9. Endo agar plates.
10. E.M.B. plates.
11. Russell double sugar agar slants.

B. Procedure:

1. Make a Gram stain of each of the organisms used.
2. Inoculate each organism into the following sugar media:
 - Glucose broth.
 - Lactose broth.
 - Sucrose broth.
 - Inositol broth.
 - Mannitol broth.
 - Xylose broth.
 - Russell double sugar agar.
3. Inoculate each organism into a tube of litmus milk.

¹In Durham fermentation tubes.

4. Inoculate each organism onto Endo agar and E.M.B. as described in Exercise 85.
5. Incubate at 37° C.

C. Examination and Record :

1. Record sugar fermentations, 24 to 48 hour incubation.
2. Record litmus milk reaction, coagulation, and curd after 2 and 7 days.
3. Record color and appearance of colonies on Endo and E.M.B. agar.
4. Record observations on Russell double sugar agar.
5. Make dichotomous key to the organism studied.

Ref.: Jordan, E. O. 1930. *Food Poisoning and Food Borne Infection*. Chicago University Press.

EXERCISE 106

DETECTION OF FOOD POISONING AND TYPHOID-DYSENTERY GROUP OF BACTERIA IN SUSPECTED FOODS OR FECES

A. Materials Needed :

1. Petri dishes containing Endo or eosine methylene blue or china blue rosolic acid agar.
2. Sterile glass rods.
3. Samples of suspected food or feces.
4. Broth in tubes.
5. Russell double sugar agar.
6. Various carbohydrate media.
7. Sera,—specific for members of the paratyphoids, typhoid, dysentery, etc., groups of bacteria.

B. Procedure :

1. Take a small amount of the suspected food (or feces) and place it in a tube of broth. Emulsify by means of a heavy platinum loop and incubate at 37° C. for 20 minutes to an hour.
2. Place a small loop of the suspension in center of petri dish containing a differential agar medium and smear over the surface by means of the glass rod. Smear a second and a third petri dish with the same glass rod. (The second or third dish will usually yield well isolated colonies.)
3. Incubate at 37° C. for 24 hours.

C. Examination and Record :

1. Examine plates and fish non-acid colonies (which resemble typhoid, etc.) onto the Russell double sugar agar.

2. Incubate for 24 hours. If there is acid and gas in the butt, and acid on the slant, the organism is probably *Esch. coli*, and may be discarded.

If the slant is alkaline, but acid and gas are present in the butt, the organism is probably a paratyphoid or other member of the intermediate group.

If there is acid but no gas in butt and slant is alkaline the organism is probably a typhoid or dysentery strain.

3. Ascertain the nature of the isolated organisms by inoculating into various sugar media and agglutination with specific antisera.

Ref.: Savage, W. G. 1920. *Food Poisoning and Food Infection*. Cambridge University Press.

Levine, Max; Ajwani, G. A.; and Weldin, J. C. 1925. "The Morgan Group of Paratyphoids." *Am. Jour. of Pub. Health*, **25**, 17-21.

Wadsworth, A. 1927. *Standard Methods of Division of Laboratories and Research*. N.Y. State Dept. of Health, pp. 156-176. Saunders, Philadelphia.

EXERCISE 107

MACROSCOPIC COLONY COUNT OF MILK (A.P.H.A.)

A. Materials Needed:

1. Petri dishes.
2. Pipettes (1 c.c.).
3. Dilution water (99 c.c. in crowned or glass-stoppered bottles).
4. Milk samples.
5. Nutrient agar (pH 6.2-7.0, preferably pH 6.6).

B. Procedure:

1. Inoculate duplicate plates with the desired quantities of the milk sample (1/10, 1/100, 1/1000, 1/10,000 c.c., etc.). The samples and dilution must be shaken vigorously and rapidly (25 shakes, of an excursion of about one foot in 7 seconds) before plating.
2. Add about 10 c.c. of nutrient agar to each petri dish (preferably in less than 15 minutes and not over 20 minutes after dilution).
3. Incubate for 48 hours at 37° C.

C. Examination and Record:

1. Count colonies with aid of a lens magnifying $2\frac{1}{2}$ diameters (opticians $3\frac{1}{2}\times$ lens).
2. Record as "colonies per c.c." or "standard plate count" per c.c.

Ref.: Standard Methods of Milk Analysis. 1927. A.P.H.A.

EXERCISE 108

METHYLENE BLUE REDUCTION TEST IN MILK

A. Materials Needed:

1. Sterile test tubes.
2. Pipettes (10 c.c. and 1 c.c.).
3. Water bath (37° C.).
4. Milk samples.
5. Standard methylene blue tablet solution (1 tablet dissolved in 200 c.c. sterile distilled water).

B. Procedure:

1. Place 10 c.c. of each milk sample in a separate tube.
2. Add 1 c.c. of the methylene blue solution to each tube of milk.
3. Place tubes in water bath and record loss of color after 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours.
4. Discuss relation of reductase test to standard plate count, as to function, advantages and disadvantages, correlation, etc.

Ref.: Standard Methods of Milk Analysis. 1927. A.P.H.A.

Fred, E. B. 1912. "A Study of the Quantitative Reduction of Methylene Blue by Bacteria Found in Milk and the Use of This Stain in Determining the Keeping Quality of Milk." *Centralb. f. Bakt.* II, **35**, 391-428.

EXERCISE 109

MICROSCOPIC COLONY COUNT OF MILK

(Frost Method)

A. Materials Needed:

1. Microscope slides.
2. Pipette calibrated so as to be able to deliver 1/20 c.c. portions.
3. Melted nutrient agar at 42–45° C.
4. Thionine stain (1 gram thionine in 1200 c.c. of hot distilled water, filtered, then 60 c.c. of glacial acetic acid added to filtrate).
5. Hot plate at 45° C.
6. Platinum loop.

B. Procedure:

1. Mark an area of 4 sq. cm. on the microscope slide.
2. Place 1/20 c.c. of the milk sample (or an appropriate dilution of the sample), on the slide, add 1/20 c.c. of the melted agar at about 45° C., and distribute evenly over the 4 sq. cm. (with a loop) and allow to harden by removing from warm plate.
3. Incubate in moist sterile chamber at 37° C. for 8 to 12 hours.

C. Examination and Record:

1. Dry the "little plate" on the slide by heating at a temperature a little below boiling.
2. Immerse the dried films in the thionine stain for 10 minutes.
3. Wash in water to remove excess stain.
4. Dry as described above. (The colonies should be deeply stained, and the agar colorless.)
5. Enumerate the colonies by examining with the microscope at least 5 fields.

Note: The factor for transforming colonies per field to colonies per c.c. must be determined for each microscope and combination of lenses used. Roughly the factors would be as follows for a 10× ocular:

OBJECTIVE	FACTOR
16 mm.	4,000
4 mm.	100,000
1.9 mm.	400,000

Ref.: Frost, W. D. 1917. "Counting the Living Bacteria in Milk."
Jour. of Bact., **2**, 567-583.

EXERCISE 110

MICROSCOPIC COUNT OF BACTERIA IN MILK

(Breed Method)

A. Materials Needed:

1. Capillary pipette discharging 0.01 c.c.
2. Microscope slides (preferably $2 \times 4\frac{1}{2}$ inches).
3. Guide plate with 1 sq. cm. areas marked thereon.
4. Milk or cream sample.
5. Ocular micrometer with circular aperture.

B. Procedure:

1. Place 0.01 c.c. of milk or cream sample on microscope which is placed on guide plate and spread as evenly as possible over 1 sq. cm. by means of a stiff platinum needle.
2. Dry film in a warm place protected against dust and flies (must be accomplished in 5 to 10 minutes).
3. Immerse dried slides in xylol for one minute or more to remove fat; drain and dry.
4. Immerse in 90% alcohol for one or more minutes.
5. Stain with alkaline methylene blue (30 c.c. saturated alcoholic methylene blue in 100 c.c. 0.01% KOH).
6. Rinse in water and if overstained decolorize with alcohol until background of film is a faint blue. (Slides may be decolorized and restained if necessary).
7. Dry and examine at once or after storage, as convenient, with oil immersion objective.

Note: If the eye piece is supplied with a micrometer which has a circular aperture and the microscope adjusted so that the diameter of this aperture is 0.146 mm. each field examined will represent 1/600,000 c.c. of milk.

8. Record the number of bacteria and the number of groups (isolated bacteria and clumps) of bacteria in at least 30 fields.
9. Compare this microscopic count with the standard plate count and discuss their relative usefulness, correlation, etc.

Ref.: Breed, R. S. 1911. "Determination of the Number of Bacteria in Milk by Direct Microscopic Examination." *Centlb. f. Bakt.* II, **30**, 337-340.

Standard Methods of Milk Analysis. 1927. A.P.H.A.

EXERCISE 111

DETECTION AND ESTIMATION OF COLON GROUP IN MILK

A. Materials Needed

1. Lactose gentian violet broth in Durham fermentation tubes.
2. Lactose brilliant green bile in Durham fermentation tubes.
3. Lactose broth in Durham fermentation tubes.
4. Eosine methylene blue agar or Endo agar plates.
5. Milk samples.

B. Procedure:

1. Inoculate various quantities of milk samples into the different liquid media.
2. Incubate at 37° C. (48 hours).

C. Examinations and Record:

1. Record any evidence of gas production after 24 hours and 48 hours.
2. If gas is present plate out, on first observation of gas, onto eosine methylene blue or Endo agar and examine for presence of colon group as in Exercises 82 to 85.
3. Compare value of the various enrichment media for isolation of colon group from milk.

Ref.: Slack, A. J., and Maddeford, C. W. 1932. "The *B. coli* Content of Raw and Pasteurized Milk." *Am. Pub. Health Jour.*, **23**, 574-578.

EXERCISE 112

EXAMINATION OF FLOUR FOR ORGANISMS RESPONSIBLE FOR ROPY BREAD

A. Materials Needed:

1. Sterile dry large potato tubes (about 150 to 200x25 mm.).
2. Sterile tap water.
3. Loaves of bread.
4. Flour samples.

B. Procedure:

1. Place 3 to 5 c.c. of sterile tap water into the constricted part of the tubes (10 or more).
2. Place a piece of bread about 2 inches by 1/2 inch into each tube, and moisten with a few drops of sterile tap water.
3. Sterilize in the autoclave.
4. Suspend 5 grams of flour in 20 c.c. of water, and heat in boiling water for about 15 minutes.
5. Inoculate tubes of bread with 2 drops to 3 c.c. of the flour suspension.
6. Incubate at about 30° C. for 48 hours.
7. Examine bread for evidence of ropiness.

Ref.: Tanner, F. W. 1932. *Microbiology of Foods*, pp. 349-366.

EXERCISE 113

BACTERIOLOGICAL EXAMINATION OF FLOUR

A. Materials Needed:

1. Nutrient agar.
2. Petri dishes.
3. Sterile water (30 c.c. in flask).
4. Flour sample.

B. Procedure:

1. Suspend 10 grams of flour in 30 c.c. of water in flask or bottle.
2. Heat to about 90° C. and place in boiling water for about 15–20 minutes.
3. Plate 1 c.c. portions of heated material on nutrient agar.
4. Incubate at 30° to 37° C. for 24 hours.
5. Count number of colonies developing.

Ref.: Tanner, F. W. 1932. *Microbiology of Foods*, pp. 349–366.

EXERCISE 114

BACTERIOLOGICAL EXAMINATION OF SUGAR FOR THERMOPHILIC-CANNED-FOODS- SPOILAGE-ORGANISMS

(Cammeron Method Modified)

A. Materials Needed :

1. Sugar samples (not less than 1/2 lb. from each of 5 bags or barrels of batch to be tested).
2. Bottles or flasks with 100 c.c. and 120 c.c. graduations.
3. Dextrose brom-cresol-purple tryptophane agar (tryptophane broth + 0.5% dextrose + 1.5% agar + 0.004% brom cresol purple).
4. Agar-agar (2%) in distilled water (sterile in tubes).
5. Liver broth in large tubes (20-25 c.c. per tube).
6. Proteose peptone ferric citrate agar in large tubes (20-25 c.c. per tube).
7. Sterile pipettes 1 c.c. and 10 c.c. graduated in 0.1 c.c.
8. Sterile water.
9. Sterile paraffine oil.

B. Procedure :

1. Estimation of flat sour spores.

- a. Place 20 grams of a sugar sample into the sterile flask, add sterile water to the 120 c.c. mark, boil for 5 minutes and replace loss by evaporation, with sterile water.
- b. Place 2 c.c. of sugar solution "a" above into each of 5 petri dishes, add dextrose brom-cresol-purple agar, mix, and allow to solidify.
- c. Incubate at 55° C. for 48 hours (incubator must be humid).
- d. Count the number of acid colonies on each plate. The combined count represents the "flat sour" organisms per 12 $\frac{2}{3}$ grams of sugar. Multiply by

6 to obtain number of spore forming "flat sour" organisms per 10 grams.

- e. Repeat "a," "b," "c," and "d" above with each of the five sugar samples.

2. Estimation of thermophilic anaerobes.

- a. Place 4 c.c. of the sugar sample B-1-a above into each of six tubes of freshly heated liver broth (this represents testing 4 grams of sugar).
- (1) Stratify with about 5 c.c. of the agar-agar solution and after solidification 2 or 3 c.c. of sterile paraffine may be added (if desired) to protect the agar seal against drying.
 - (2) Incubate at 55° C. for 72 hours.
 - (3) Record acid production, gas (as evidenced by raising or splitting the agar) in each tube.
- b. Repeat 2-a above with each of the five sugar samples.

3. Estimation of sulphide spoilage organisms.

- a. Place 4 c.c. of the sugar sample B-1-a above into each of six tubes of proteose-peptone ferric citrate agar, which have been freshly boiled to expel air.
- b. Incubate at 55° C. for 72 hours.
- c. Record number of black colonies.

C. Records:

Note: Cammeron recommends the following standards.

Flat sour spores. For the five samples examined, there shall be a maximum of not more than 75 spores, and an average of not more than 50 spores per 10 grams of sugar.

Thermophilic anaerobic spores. These shall be present in not more than three (60%) of the five samples and in any one sample to the extent of not more than four (65%) of the subsamples.

Sulphide spoilage spores. These shall be present in not more than two (40%) of the five samples, and in any one sample to the extent of not more than five spores per 10 grams. This would be equivalent to two colonies in the six inoculated tubes of each sample of sugar tested.

1. Tabulate the results for each of the samples of sugar examined.
2. In what respects does the above procedure differ from the standard procedure of Cammeron?
3. Does sugar examined conform to proposed standards?

Ref.: Cammeron and Yesair. March 21, 1931. "Sugar Contamination; Its Effect in Canning Corn." *The Canner*.

James, L. H. 1930. "A Sugar Tolerant Member of the *Colony-aerogenes* Group." *Jour. of Bact.*, **19**, 145-148.

Tanner, F. W. 1932. *Microbiology of Food*, pp. 394-411.

APPENDICES

APPENDIX A

PREPARATION OF STANDARD AND SPECIAL CULTURE MEDIA

A. Nutrient Agar (S.M.W.A.) ¹

1. Add 3 g. of beef extract, 5 g. of peptone, and 15 g. of agar (undried market product as stored in the ordinary laboratory cupboard), to 1000 c.c. of distilled water. Boil until all the agar is dissolved.
2. Make up the lost weight with hot distilled water and adjust the reaction so that the pH value, after the final sterilization, will be between 6.4 and 7.0.
3. Bring to a boiling temperature, stirring frequently, restore the lost weight with hot distilled water and clarify.
4. Distribute in the desired containers and sterilize as directed under sterilization.

Note: Sterilization. All media shall be sterilized in the autoclave at 15 lbs. (120° C.) for 15 minutes after the pressure has reached 15 lbs. All air must be forced out of the autoclave by allowing live steam to stream through it for a few minutes before the pressure is allowed to rise. As soon as possible after sterilization the medium shall be removed from the autoclave and cooled rapidly. Rapid and immediate cooling of gelatin and all sugar media is imperative.

Media shall be sterilized in small containers and these must not be closely packed together. No part of the medium shall be more than 2.5 centimeters from the outside surface of the glass, or from the surface of the medium.

B. Nutrient Gelatin (S.M.W.A., 1933)

1. Add 3 g. of beef extract, 5 g. of peptone, and 120 g. of gelatin (undried market product as stored in the ordinary laboratory cupboard), to 1000 c.c. of distilled water.

¹Standard Methods of Water Analysis of the American Public Health Association, and American Water Works Association, 1933.

2. Heat slowly on steam bath of 65° C. until all the ingredients are dissolved.
3. Make up the lost weight with distilled water and adjust the reaction so that after the final sterilization the pH value will be between 6.4 and 7.0.
4. Bring to a boil, stirring vigorously. Make up the lost weight with distilled water and clarify.
5. Distribute in the desired containers and sterilize as directed under sterilization.¹

C. Nutrient Broth (S.M.W.A., 1933)

1. Add 3 g. of beef extract and 5 g. of peptone to 1000 c.c. of distilled water.
2. Heat slowly on a water bath to 65° C., stirring until dissolved.
3. Make up the lost weight with distilled water and adjust the reaction so that the final pH will be between 6.4 and 7.0.
4. Bring to a boil over a free flame, cool to 25° C., make up the lost weight with distilled water and clarify.
5. Distribute in test tubes, 10 c.c. to each tube, or in other desired containers.
6. Sterilize as directed under sterilization.¹

D. Sugar Broths (S.M.W.A., 1933)

Sugar broths shall be prepared in the same general manner as nutrient broth with the addition of 0.5% of the required carbohydrate. The removal of muscle sugar is unnecessary as the beef extract and peptone are free from any fermentable carbohydrates. The reaction of sugar broths shall be the same as that required for nutrient broth.² Sterilization shall be in the autoclave at 15 lbs. for 15 minutes after the pressure has reached 15 lbs., provided that the total time of exposure to any heat is not more than one-half hour.³ If it is not possible to limit the exposure to heat to one-half hour or less, then a 10 or 20% solution of the required sugar shall be made in distilled water and sterilized in the autoclave for 15 minutes after the pressure has

¹ See note under Nutrient Agar.

² The committee of the Society of American Bacteriologists recommends 1.0% carbohydrate and a reaction of pH 7.0.

³ Twelve pounds pressure followed by rapid cooling and incubation to detect contaminated tubes has been found very serviceable at this laboratory.

reached 15 lbs., or by heating in the Arnold sterilizer at 100° C. for 1½ hours. This solution shall then be added to sterile nutrient broth in amounts sufficient to make a 0.5% solution of the carbohydrate and the mixture shall then be tubed with proper precautions for preserving its sterility and sterilized at 100° C. for 30 minutes. Or it is permissible to add by means of a sterile pipette directly to a tube of sterile nutrient broth enough of the sugar solution to make the required 0.5% concentration. The tubes so made shall be incubated at 37° C. for 24 hours as a test for sterility before they are used.

E. Synthetic Carbohydrate Media (S.A.B.)

Monobasic ammonium phosphate	1.0 g.
Potassium chloride	0.2 g.
Magnesium sulphate	0.2 g.
Water (distilled)	1000 c.c.
Sugar or other carbon source	10.0 g.

(For use as solid medium add 15 g. agar per liter).

Adjust reaction to pH 7 (about 6 c.c. normal NaOH per liter required).

Appropriate indicators may be added.

F. Loeffler's Serum ¹

(This medium is particularly adapted to diagnosis of diphtheria)

The blood of cattle should be collected in large pans or pails at the abattoir. This vessel of blood should then be kept in the cold-storage room and the next morning the more or less clear serum will have been squeezed out from the clot. Collect this serum and keep in the ice chest for future use. If to be kept for a long time, it is advisable to add about 2% of chloroform to the serum in tightly corked flasks. This will not only keep the serum, but will eventually sterilize it.

To make Loeffler's serum, take one part of glucose bouillon and three parts of blood-serum. Mix, tube, and coagulate the albumen in the inspissator or rice cooker, giving the tubes a proper slant before heating. Sterilize in the autoclave as previously directed (7 lbs.) or in the Arnold on three successive days.

¹Stitt's *Practical Bacteriology, Blood Work and Animal Parasitology*.

G. Tryptophane Broth (A.P.H.A., 1925)

(A special medium for the indol test)

To 1000 c.c. of distilled water add 0.3 gram tryptophane, 5 grams dipotassium hydrogen phosphate (K_2HPO_4), and 1 gram peptone. Heat until ingredients are thoroughly dissolved, tube (6 to 8 c.c.), and sterilize in the autoclave for 15 minutes after the pressure reaches 15 pounds. Some American peptones are standardized to contain a uniform amount of tryptophane. If such peptone is used the tryptophane in the above formula may be omitted and the peptone increased to 5 grams.

H. Potatoes

1. Obtain good sized potatoes and clean them carefully.
2. With a potato borer cut cylinders out of the potato and with a knife cut off the skin at right angles to the long axis of the cylinder. Keep these cylinders under water to prevent oxidation.
3. Cut cylinders diagonally so as to give a slanting surface and place each piece thus obtained, base downward, into a potato tube.

Note: Potato tubes are test tubes with a constriction about one inch from the bottom. They should contain about one-fourth inch of water.

4. Sterilize in the autoclave at 15 pounds for 20 minutes.

I. Plain and Litmus Milk

1. Adjust reaction of skim milk to neutrality to phenol red or +1.0 to phenolphthalein.
2. Add 2% of 1% azolitmin or litmus solution. The color should be violet with a tendency to blue. If this coloration is not distinct more of the indicator should be added up to 5 to 8%. If too acid or alkaline adjust with N/1 acid or alkali to neutrality.
3. Sterilize in the Arnold for 30 minutes on three successive days. (If necessary the autoclave may be used at 15 pounds for 10 to 15 minutes.)

Note: Plain milk prepared in the same way without the addition of the litmus is also used as a medium.

J. Dunham's Solution

1. Place in a sauce pan:

10 g. peptone.
 5 g. NaCl.
 1000 c.c. distilled water.

2. Bring to a boil over the free flame. Boil for 10 minutes. Make up loss due to evaporation.
3. Filter through filter paper until clear.
4. Fill tubes one-third full.
5. Sterilize in the autoclave at 15 pounds for 15 minutes.

K. Clark and Lubs Medium

(For methyl red test)

Peptone (Witte).....	5 g.
Dextrose (anhydrous).....	5 g.
Dipotassium phosphate.....	5 g.
Water (distilled).....	1000 c.c.

Heat till ingredients are dissolved, distribute in tubes, and sterilize in autoclave at 15 lbs. for 15 minutes.

Note: If "Witte" peptone is not available 7 g. of Difco Proteose Peptone may be employed, and the reaction adjusted to pH 6.9-7.0.

L. Brain Medium (S.A.B.)

1. Boil sheep brains with an equal volume of distilled water.
2. Decant water (save) and press brains through a fruit press or potato ricer.
3. Add to the pressed brains
 - a. the decanted water,
 - b. 2% peptone,
 - c. 0.1% dextrose,
 and mix thoroughly.
4. Tube by punching through the filling funnel with a glass rod, filling tubes about half full.
5. Place tubes loosely in wire baskets to facilitate sterilization.
6. Sterilize five successive days in Arnold for 30 minutes. (Autoclaving is permissible if blowing up of media can be avoided.)

Note: A meat medium may be prepared as above.

M. Nitrate Broth

1. Place in a sauce pan:

1.0 g. peptone (NO₂ free).
 0.5 g. NaCl.
 0.2 g. KNO₃.
 1000 c.c. H₂O (NO₂ free).

2. Boil, filter through filter paper, tube and sterilize in the autoclave at 15 pounds for 15 minutes.

Note: This may be prepared by adding 0.2 g. KNO₃ to 100 c.c. Dunham's solution and diluting to one liter.

N. Synthetic Nitrate Medium (S.A.B.)

K ₂ HPO ₄	0.5 g.
CaCl ₂	0.5 g.
MgSO ₄	0.2 g.
Glucose.....	10.0 g.
KNO ₃	1.0 g.
Water (distilled).....	1000 c.c.

O. Endo (Fuchsin Sulphite) Agar (S.M.W.A.)

(A special medium for detection and isolation of bacteria of the colon-typhoid group)

1. *Preparation of Stock Agar.*

- a. Add 5 g. of beef extract, 10 g. of peptone, and 30 g. of agar (undried market product as stored in the ordinary laboratory cupboard), to 1000 c.c. of distilled water.
- b. Boil until all the agar is dissolved and then make up the lost weight due to evaporation, with distilled water.
- c. Titrate and adjust the reaction so that the final pH value will be between 7.8 and 8.2. This agar may then be clarified sufficiently by either one of the following procedures. (Inasmuch as a 3% agar is rather difficult to filter and as this particular medium does not have to be entirely free of precipitate, procedure (2) is probably the better one to employ.)

Procedure (1): Bring to boil over a free flame, stirring constantly, and filter through cotton or cloth.

Procedure (2): Place a straight walled container holding the agar in the autoclave and hold at 15 lbs. pressure for 15 minutes. Shut off the steam and let the agar stand in

the autoclave until solidification is complete (over night if possible). Remove the container from the autoclave, dump the solidified agar on clean paper and cut off the detritus from the bottom and discard. Cut and melt the clear supernatant agar and distribute in 100 c.c., or larger known quantities, in flasks large enough to hold the other ingredients which are to be added later.

Sterilize in the autoclave at 15 lbs. for 15 minutes after the pressure reaches 15 lbs.

2. Preparation of Endo Medium Plates.

- a. Prepare a 10% solution of basic fuchsin in 95% alcohol, allow to stand 24 hours, decant, and filter the supernatant fluid. This is a stock solution. Certified basic fuchsin must be used.
- b. When ready to make plates melt a known portion of the stock agar and to each 100 c.c. of agar add the following ingredients in the order given, mixing thoroughly after the addition of the final reagent:
- c. One per cent of chemically pure lactose in sterile solution, 0.5 c.c. of the stock basic fuchsin solution (10% alcoholic solution) and 0.125 g. anhydrous C.P. sodium sulphite dissolved in a small amount of hot distilled water. The sulphite solution must be made up fresh each time. Mix thoroughly.
- d. Pour plates and allow to harden in the incubator before use. Inoculate by streaking on the surface.

P. Simplified Endo (Fuchsin Sulphite) Agar (Levine)

1. Place in pan:

Distilled water	1000 c.c.
Peptone (Difco)	10 g.
Dipotassium phosphate (K_2HPO_4)	3.5 g.
Agar	15-30 g. (as desired)

2. Boil until dissolved and make up loss due to evaporation with distilled water.
3. Place measured quantities (100 or 200 c.c.) in flasks or bottles and sterilize at 15 lbs. 15 to 20 minutes.
4. For use add to each 100 c.c. of the above melted agar:

Lactose	1 gram (or 5 c.c. of 20% solution)
Basic fuchsin (pararosanilin and rosanilin) (saturated alcoholic solution)	0.5 c.c.
Sodium sulphite (freshly prepared 10% solution)	2.5 c.c.

No adjustment of reaction is made and filtration is not necessary if the medium is to be used for streak or plate cultures.

Q. Litmus Lactose (Wurtz) Agar (A.P.H.A., 1925)

(For the detection and isolation of lactose fermenters)

Litmus-lactose-agar shall be prepared in the same manner as nutrient agar with the addition of 1% of lactose just before sterilization. The reaction shall be a faint pink with phenol red, or, if, on titration with phenolphthalein, the reaction is not already between neutral and +1, adjust to neutral. One c.c. of sterilized litmus or azolitmin solution shall be added to each 10 c.c. of the medium just before it is poured into the petri dish, or the mixture may be made in the dish itself.

R. Eosine Methylene Blue Agar (Levine)

(A special medium for differentiation and isolation of the colon-typhoid group)

1. Place in a sauce pan or flask:

Distilled water	1000 c.c.
Peptone (Difco)	10 g.
Dipotassium phosphate (K_2HPO_4)	2 g.
Agar	15 g.

2. Boil ingredients until dissolved and make up any loss, due to evaporation, with distilled water.
3. Place measured quantities (100 or 200 c.c.) in flasks or bottles, and sterilize in the autoclave at 15 pounds pressure for 15 to 20 minutes.
4. Just prior to use add, to each 100 c.c. of the melted agar prepared as above, the following:

Lactose, sterile 20% sol.	5 c.c.
Eosine yellowish 2% aqueous sol.	2 c.c.
Methylene blue 0.5% aqueous sol.	2 c.c. ¹

¹American samples of methylene blue have been found to contain a higher concentration of dye than the old Grüber stain, and it may therefore be necessary to reduce the quantity of methylene blue for best results. The medium should be wine colored when cooled.

5. Pour medium into petri dishes, allow to harden, and inoculate by streaking on the surface.

There is no adjustment of the reaction and filtration of the medium is not necessary.

It is allowable to add all ingredients to the stock agar at the time of preparation, place in tubes or flasks and sterilize. Decolorization occurs during sterilization, but the color returns on cooling.

S. Yeast Water

1. Place 75 grams compressed yeast in one liter of water.
2. Bring to a boil over an asbestos pad for 30 minutes.
3. Filter through filter paper.
4. Distribute in tubes or flasks.

T. Beer Wort Media

1. *Preparation of malt.*
 - a. Soak good clean barley in warm water, and keep in a warm place until the sprouts are about $\frac{2}{3}$ the size of the barley grains. (If some of the sprouts are $\frac{1}{2}$ to 1 inch long, germination is far enough along to yield a good malt.)
 - b. Drain, dry thoroughly, and grind.
2. *Preparation of wort.*
 - a. To 1000 grams of malt, prepared as above, add 2500 c.c. of water at 60° to 70° C. and soak for 1 hour.
 - b. Drain off the liquid into clean flasks or other containers.
 - c. Add an additional 2500 c.c. of water (60° to 70° C.) to the malt and soak for 1 hour.
 - d. Drain off the liquid and add to previous drainings.
 - e. Autoclave. (15 pounds for 15 minutes.)
 - f. Filter to remove precipitate.
 - g. Distribute filtrate in test tubes or other containers and again autoclave. (15 pounds for 15 minutes.)
3. *Preparation of wort agar.*
 - a. Dilute the wort prepared as above with an equal volume of water.
 - b. Boil for 15 minutes, make up loss and filter.
 - c. Add 20 grams of agar per liter, boil till dissolved.
 - d. Sterilize at 10 pounds for 15 minutes.

U. Malt Extract Broth and Agar

This is an excellent substitute for beer wort.

1. Dissolve the following:

Malt extract (Difco)	15 g.-30 g.
K_2HPO_4	1 g.
NH_4Cl	1 g.
Citric acid N/1	15 c.c.
Water, distilled	1000 c.c.

2. Sterilize at 15 lbs. for 15 minutes.

To make medium solid add 20 grams of agar-agar to malt extract broth and sterilize at 15 lbs. for 10 to 15 minutes.

V. Carrot Juice Calcium Sulphate Agar

1. Grind or scrape very finely 1000 grams of carrots.
2. Add 200 c.c. of distilled water and boil for 10 minutes; make up loss.
3. Filter through two or three layers of cheese cloth and squeeze out as much juice as possible.
4. Add 2% agar and 1% $CaSO_4$; boil till agar is dissolved; make up loss.
5. Tube and sterilize at 15 lbs. 15-20 minutes.

W. Hydrogen Sulphide Medium

Difco proteose peptone	20 g.
K_2HPO_4	1 g.
Ferric citrate	0.5 g.
Agar	15 g.
Water	1000 c.c.

Dissolve by boiling, tube and sterilize at 15 lbs. for 15 minutes.

X. Prepared Dehydrated Media

Of the media employed in this manual the following may be obtained in dehydrated form ready for use after simple solution and sterilization:

Bacto nutrient broth.
 Bacto nutrient agar.
 Bacto nutrient gelatin.
 Bacto dextrose broth.

Bacto lactose broth.
 Bacto litmus milk.
 Bacto purple milk.
 Bacto tryptophane broth.
 Russell double sugar agar.
 Levine's eosine methylene blue agar.
 Bacto Endo agar.
 Bacto malt extract broth.
 Bacto malt extract agar.
 Bacto Loeffler's blood-serum.
 Bacto brilliant green bile (2% and 5%).
 Bacto neutral red medium.
 Bacto MacConkey's agar.
 Bacto Loeffler's blood-serum.
 Bacto peptone iron agar.

Y. Physiological Salt

(Water blanks)

Water blanks are tubes or flasks or bottles containing definite quantities of physiological salt solution.

1. Prepare physiological salt solution by adding 8.5 grams of NaCl to 1000 c.c. of distilled water.
2. Place in each of ten test tubes 9.5 c.c. of the salt solution. On sterilization about 0.5 c.c. will evaporate thus leaving 9 c.c., which on addition of 1 c.c. of the substance to be diluted will yield a dilution of 1 to 10.
3. To flasks or bottles add 103 c.c. of physiological salt solution. This will lose about 4 c.c. upon sterilization and will yield on addition of 1 c.c. of the material to be diluted, a dilution of 1 to 100.
4. Sterilize in the autoclave at 15 pounds for 20 minutes.

Z. Plaster Paris Blocks

1. Take large corks and roll a piece of note paper around each so that about one-half inch projects above the broader base of the cork. Make secure with two pins.
2. Fill each of the molds thus formed with plaster paris which has been made into a thick paste by mixing with distilled water.
3. When hard, remove the paper and place the plaster paris blocks in tall petri dishes.
4. Sterilize in the dry oven, 150° C. for two hours.

APPENDIX B

PREPARATION OF STAINING SOLUTIONS AND SPECIAL STAINING METHODS

A. General Stains

Stock solutions consist of saturated alcoholic or aqueous solutions of the dyes.

a. Saturated alcoholic solutions:

Basic fuchsin 3.0 grams in 100 c.c. alcohol (95%).
Methylene blue 7.0 grams in 100 c.c. alcohol (95%).
Gentian violet 4.8 grams in 100 c.c. alcohol (95%).

b. Saturated aqueous solutions:

Methylene blue 6.7 grams in 100 c.c. water (distilled).
Gentian violet 1.5 grams in 100 c.c. water (distilled).

In preparing saturated solutions it is best to employ an excess of the commercial dye (at least 50% excess) to insure saturation, and filter the solution before use.

Saturated solutions are not efficient for staining; they must be adequately diluted.

a. *Methylene Blue*

Methylene blue (saturated alcoholic solution)	5.00 c.c.
Distilled water	95.00 c.c.

b. *Gentian Violet*

Gentian violet (saturated alcoholic solution)	5.00 c.c.
Distilled water	95.00 c.c.

c. *Anilin Gentian Violet*

(Emended statement. Committee on Manual, S.A.B.)

SOLUTION A

Crystal violet (85% dye content)	2.5 g.
Ethyl alcohol (95%)	12 c.c.

STAINING SOLUTIONS AND METHODS 269

SOLUTION B

Anilin	2 c.c.
Distilled water	98 c.c.

Shake and allow to stand for a few minutes, then filter.
Mix solutions A and B.

d. *Stabilized Gentian Violet* (Kilduffe)

SOLUTION A

Formalin	5 c.c.
Distilled water	95 c.c.

SOLUTION B

Saturated alcoholic gentian violet.

Mix:

25 c.c. of solution B.

75 c.c. of solution A.

e. *Carbol Fuchsin* (Ziehl)

Basic fuchsin (saturated alcoholic solution)	10.0 c.c.
Phenol (5.0%)	100.0 c.c.

The stain may also be prepared as follows:

Basic fuchsin	1.0 g.
Alcohol (95%)	10.0 c.c.

Shake thoroughly, then add:

Phenol (5.0%)	100.00 c.c.
-------------------------	-------------

Shake the mixture, allow to stand several days, shaking occasionally, and filter.

The Committee on Manual of Methods of the S.A.B. recommends the following emended statement of formula:

SOLUTION A

Basic Fuchsin (90% dye content)	0.3 g.
Ethyl alcohol (95%)	10 c.c.

SOLUTION B

Phenol	5 g.
Distilled water	95 c.c.

Mix solutions A and B.

f. Kinyoun's Carbol Fuchsin

Basic fuchsin	4 g.
Phenol crystals	8 g.
Ethyl alcohol (95%)	20 c.c.
Distilled water	100 c.c.

The following five stains are frequently used as contrast or counterstains.

g. Eosine

Yellowish (water soluble) eosine	1.0 g.
Distilled water	100.0 c.c.

Dissolve, then add:

Alcohol (95%)	5.0 c.c.
-------------------------	----------

h. Safranin

Safranin	0.5 g.
Distilled water	100.0 c.c.

i. Bismark Brown (Vesuvium)

Bismark brown	0.5 g.
Distilled water	100.0 c.c.

j. Dilute Carbol Fuchsin

Carbol fuchsin	10.0 c.c.
Distilled water	100.0 c.c.

k. Picric Acid

Saturated solution of picric acid in distilled water.

B. Special Stains and Staining Methods**1. GRAM STAIN***a. Buchanan's Method***SOLUTION 1**

Gentian violet (saturated alcohol solution)	6 c.c.
Anilin water	50 c.c.

STAINING SOLUTIONS AND METHODS 271

(To prepare anilin water shake 5 c.c. of anilin oil in 125 c.c. of distilled water and filter through filter paper which has been thoroughly wetted with distilled water.)

SOLUTION 2

(Lugol's Solution)

Iodine.....	1 g.
Potassium iodide.....	2 g.
Water (distilled).....	300 c.c.

Stain for 1 to 2 minutes with the anilin oil gentian violet, then for 1 to 2 minutes with Lugol's solution. Decolorize with 95% alcohol until no more color is removed. Counterstain with safranin.

b. *Carbol Gentian Violet* (Nicolle)

The following is recommended for the Gram stain in place of anilin gentian violet by the Committee on Manual, S.A.B.

SOLUTION A

Crystal violet (85% dye content).....	2 g.
Ethyl alcohol (95%).....	10 c.c.

SOLUTION B

Phenol.....	1 g.
Distilled water.....	100 c.c.

Mix solutions A and B.

c. *Sterling's Method*

The anilin oil gentian violet is prepared in the following manner:

Gentian violet.....	5 g.
Alcohol (95%).....	10 c.c.
Anilin.....	2 c.c.
Water (distilled).....	88 c.c.

Add the anilin oil water slowly to the gentian violet while grinding and then filter the solution.

The anilin oil gentian violet is much more stable when prepared in this way.

For staining, treat with anilin oil gentian violet 1 minute, Lugol's solution 1 minute, and decolorize with 95% alcohol for 2 minutes, then counterstain with safranin.

d. Nicolle's Method

STAINING SOLUTION

Gentian violet (saturated alcohol solution)	10 c.c.
Phenol (5.0%)	100 c.c.

DECOLORIZING AGENT

Absolute alcohol	75 c.c.
Acetone	25 c.c.

Stain with carbol gentian violet 1 to 5 minutes and treat with Lugol's solution for 4 to 6 seconds. Decolorize with alcohol-acetone. Counterstain with eosine or safranin.

e. Atkin's Method

SOLUTION A

Gentian violet (saturated alcohol solution)	25 c.c.
Anilin sulphate solution (0.1%)	75 c.c.

SOLUTION B

Iodine	2 g.
Sodium hydroxide (N/1)	10 c.c.
Water distilled	90 c.c.

Stain 1 minute with the anilin sulphate gentian violet, then 1 minute with the alkaline iodine and decolorize with 95% alcohol for 1 minute. Counterstain (with eosine, dilute carbol fuchsin or safranin).

f. Huckers. Modification of Gram Stain

(Recommended by Committee on Manual, S.A.B.)

SOLUTION A

Crystal violet (85% dye content)	4 g.
Ethyl alcohol (95%)	20 c.c.

STAINING SOLUTIONS AND METHODS 273

SOLUTION B

Ammonium oxalate.....	0.8 g.
Water (distilled).....	80 c.c.

Mix solutions A and B in equal volumes. If this mixture is too difficult to decolorize dilute solution A as much as ten times if necessary.

COUNTERSTAIN

Safranin (2.5% in 95% alcohol).....	10 c.c.
Water.....	100 c.c.

Stain one minute with the ammonium-oxalate-crystal-violet solution.

Immerse in Lugol's iodine solution for one minute, wash in water, and blot dry.

Decolorize in 95% ethyl alcohol for 30 seconds with gentle agitation.

Cover with counterstain for 10 seconds, wash, dry, and examine.

g. Kopeloff and Beerman's Modification of Gram Stain

(Recommended by Committee on Manual, S.A.B.)

SOLUTION A

Gentian or crystal violet.....	1 g.
Water (distilled).....	100 c.c.

SOLUTION B

Sodium bicarbonate....	1 g.
Water (distilled).....	20 c.c.

Just before use, mix 30 drops of solution A with 8 drops of solution B.

IODINE SOLUTION

Iodine.....	2 g.
Sodium hydroxide.....	10 c.c.

After the iodine is dissolved make up to 100 c.c. with water.

COUNTERSTAIN

Basic fuchsin.....	0.1 g.
Water (distilled).....	10 c.c.

Stain 5 minutes or more with alkaline gentian violet.

Rinse with the iodine solution, then add more iodine solution and allow to stand two minutes or longer.

Drain off iodine solution and blot dry (without washing).

Decolorize with 50% acetone in alcohol by dropping onto slide and permitting it to drain off until no color is seen to come off (generally less than 10 seconds), then dry in air.

Counterstain 10-30 seconds.

Wash in water, dry, and examine.

2. DIPHTHERIA STAINS

a. *Loeffler's Methylene Blue*

Methylene blue (saturated alcoholic solution) 30 c.c.

Potassium hydroxide (1/10,000) 100 c.c.

Prepare film in the ordinary way and stain for 3 to 5 minutes.

b. *Neisser's Stain*

SOLUTION 1

Methylene blue 0.1 g.

Absolute alcohol 20.0 c.c.

Glacial acetic acid 5.0 c.c.

Distilled water 100.0 c.c.

SOLUTION 2

Safranin or Bismark brown

Stain for 10 to 30 seconds with solution 1, wash in water and counterstain with Bismark brown or safranin for 5 to 30 seconds. (The granules stain blue, the body of the cells brown or pink.)

c. *Albert's Stain*

SOLUTION 1

Toluidin blue 0.15 g.

Methyl green 0.20 g.

Acetic acid (glacial) 1.00 c.c.

Alcohol (95%) 2.00 c.c.

Water (distilled) 100.00 c.c.

After standing for one day the solution is filtered and is ready for use.

STAINING SOLUTIONS AND METHODS 275

SOLUTION 2

Iodine.....	2.0 g.
Potassium iodide.....	3.0 g.
Water (distilled).....	300.0 c.c.

The solution is ready for use as soon as the iodine is entirely dissolved.

Stain with solution 1 for one minute, wash in water, dry with good absorbent filter paper; stain with solution 2 for one minute; wash and dry with filter paper.

Granules of diphtheria bacilli stain black, the bars dark green, and intermediate portions are light green.

3. STAINS FOR MYCOBACTERIUM TUBERCULOSIS

a. *Ziehl-Gabbett Method*

SOLUTION 1

Preparation of Gabbett's methylene blue:

Methylene blue (dry).....	2.0 g.
Sulphuric acid (sp. 1.84).....	25.0 c.c.
Distilled water.....	75.0 c.c.

SOLUTION 2

Carbol fuchsin. (See pages 269-270.)

The smear is stained with hot carbol fuchsin for 4 or 5 minutes and counterstained with Gabbett's methylene blue for 30 seconds.

The tubercle bacilli stain red; other bacteria in the field, blue.

b. *Ziehl-Nielsen Method*

Preparation of staining solution:

- (1) Carbol fuchsin (Ziehl or Kinyoun).
- (2) Plain or Loeffler's methylene blue.
- (3) Acid alcohol.

HCl (sp. gr. 1.2).....	64 c.c.
Alcohol (95%).....	1000 c.c.

Smears are stained with hot carbol fuchsin, for 4 to 5 minutes decolorized with acid alcohol, and counterstained with plain or Loeffler's methylene blue for 10 to 15 seconds.

Tubercle bacilli stain red, others blue.

c. Schulte-Tiggs Method

- (1) Flood the slide with carbol fuchsin.
- (2) Heat the slide to steaming and continue heating for one minute. (Avoid excessive heating.)
- (3) Wash with tap water, and decolorize with sodium sulphite (10% Na_2SO_3 in water which should be prepared fresh at least weekly).
- (4) Wash and counterstain with picric acid (saturated aqueous solution).
- (5) Wash in water, dry, and examine.

The tubercle bacilli are stained red, other bacteria yellow.

4. SPORE STAINS

a. Moller's Method

- (1) Prepare a smear and fix by heat.
- (2) Flood the slide with chloroform for one or two minutes.
- (3) Wash.
- (4) Treat with 5% chromic acid solution for one minute.
- (5) Wash in water.
- (6) Stain with hot carbol fuchsin for two to five minutes.
- (7) Decolorize with 1% sulphuric acid for a few seconds.
- (8) Counterstain with Loeffler's methylene blue.

The spores will stain red, vegetative cells and sporangia and outer wall of spores should stain blue.

b. Schaeffer and Fulton Method

- (1) Prepare a smear and fix by flaming three times.
- (2) Flood with malachite green (5% aqueous solution allowed to stand half hour and filtered) and heat to steaming three or four times within one-half minute.
- (3) Wash off excess stain under tap for about half minute.
- (4) Apply a 0.5% aqueous safranin solution for one-half minute.
- (5) Wash, blot, dry, and examine.

5. CAPSULE STAIN

a. The Hiss Capsule Stain

- (1) Prepare and fix smear in the ordinary way.
- (2) Stain with anilin oil gentian violet or carbol fuchsin for five seconds, or until steam arises.

- (3) Wash off the stain with 20% aqueous copper sulphate solution.
- (4) Blot and dry in air. (Do not wash.)

b. The Huntoon Capsule Stain

Method of preparing the reagents employed:

Solution 1.—To be used as a diluent.

Three grams of sodium caseinate (nutrose) are sifted into 100 c.c. of distilled water and heated to 100° C. in the Arnold sterilizer for one hour.

Add 5 c.c. of 2% aqueous solution of carbolic acid to serve as a preservative. Decant into test tubes and allow to settle.

Employ the supernatant fluid as the diluent.

(Since the supernatant liquid tends to become thinner by constant precipitation of the nutrose the solution should occasionally be reboiled.)

Solution 2.—Fixing and staining solution.

2% aqueous solution of carbolic acid	100 c.c.
Concentrated lactic acid	0.25 to 0.5 c.c.
1% acetic acid	1 c.c.
Saturated alcoholic solution of basic fuchsin	1 c.c.
Carbol fuchsin (old)	1 c.c.

(This solution must be kept tightly corked, and it will then keep very well.)

Experience has shown that in the above solution the addition of old and fresh fuchsin as given makes a better product than an increase in the amount of either one alone.

Technique of staining:

- (1) Employ the nutrose solution (no. 1) as a diluent, emulsifying the bacteria in one or two loopfuls and then spreading on the slide as thin a film as possible with the loop. The use of the edge of a slide in spreading the film, as in blood work, is not to be recommended.
- (2) Allow to dry in air. (Do not fix with heat.)
- (3) Cover the film with the fixative and staining solution (no. 2) and allow to act for thirty to forty-five seconds.
- (4) Wash quickly in water, dry, and examine.

(For capsule stain of cultures grown in milk see Exercise 15.)

6. MISCELLANEOUS STAINS

a. *Acetic Thionin Solution* (Frost)

Thionin.....	1 g.
Distilled water (hot).....	1200 c.c.

Dissolve and filter; then add:

Glacial acetic acid.....	60 c.c.
--------------------------	---------

Stain used for staining bacteria in agar films inoculated with milk.

b. *Dorner's Nigrosin Solution*

Nigrosin.....	10 g.
Distilled water.....	100 c.c.

Boil 30 minutes in an Erlenmeyer flask then add as preservative.

Formalin.....	0.5 c.c.
---------------	----------

Filter twice through double filter paper and store in test tubes (5 c.c. per tube).

Used for negative demonstration of bacteria.

Mix a loop of bacterial suspension with a loop of nigrosin on slide; dry, and examine.

c. *Wright's Blood Stain*

Methylene blue hydrochloride (90% dye content)	0.9 g.
Sodium carbonate (0.5% aqueous solution).....	100 c.c.

Heat in a steam sterilizer at 100° C. one hour (in containers in which the solution is not over 6 cm. deep).

Cool and filter.

To filtrate add:

Eosine Y (dye content about 85%).....	1 g.
Distilled water.....	500 c.c.

Mix thoroughly and filter. Save precipitate, and dissolve for use as follows:

Wright's stain (dry).....	0.1 g.
Methyl alcohol, absolute, neutral, acetone free....	60 c.c.

Allow stain to stand a day or two, then filter.
Always filter before using.

Note: Wright's stain can be obtained in dry form or solution ready for use.

d. *Giemsa's Blood Stain*

Azure II eosin	3 g.
Azure II	0.8 g.
Glycerol C.P.	250 c.c.
Methyl alcohol, neutral, acetone free.	250 c.c.

(For staining bacteria and protozoa in blood or tissue reduce glycerol content to 125 c.c. and increase methyl alcohol to 375 c.c.)

APPENDIX C

TESTS AND REAGENTS FOR METABOLIC PRODUCTS

A. Tests for Indol

1. SALKOWSKI TEST

a. Reagents required:

(1) Sulphuric acid.....	1 part
Distilled water.....	9 parts
(2) Sodium nitrite.....	0.2 g.
Distilled water.....	1000 c.c.

b. The test is made in the following manner:

About 5 c.c. of the culture is acidified with about 1 c.c. of 10% sulphuric acid, then add 1 c.c. of sodium nitrite solution so as to form a layer on the surface.

A positive reaction is indicated by the development of a pink ring at the plane of contact of the acidified culture and the sodium nitrite solution. (Reaction not specific.)

2. EHRLICH TEST

a. Reagents required:

(1) Paradimethylaminobenzaldehyde.....	2 g.
Alcohol (95%).....	100 c.c.
(2) Concentrated hydrochloric acid.	

b. Test: 1 c.c. of the paradimethylaminobenzaldehyde is added to the culture, then the hydrochloric acid is introduced, a drop at a time, until a red zone appears. (Not more than 1/2 c.c. of acid should be used.)

The red color is soluble in chloroform and this characteristic should be ascertained.

3. THE EHRLICH-BÖHME TEST

a. Reagents required:

SOLUTION A

Paradimethylaminobenzaldehyde	1.0 g.
Absolute alcohol	95.0 c.c.
HCl (sp. gr. 1.2)	20.0 c.c.

SOLUTION B

Saturated aqueous solution of potassium persulphate ($K_2S_2O_8$)

b. Technique:

To 10 c.c. of test material add 5 c.c. of solution A, then 5 c.c. of solution B.

Development of a red color in 5 minutes indicates a positive test for indol.

4. THE GORÉ TECHNIQUE

a. Reagents required:

Same as for Ehrlich-Böhme Test.

b. Technique:

- (1) Moisten a cotton plug of absorbent cotton with a few drops (4 to 6) of saturated persulphate solution, then with the paradimethylaminobenzaldehyde reagent.
- (2) Force plug into tube until moistened portion is about one inch from surface of test material.
- (3) Place test tube in a boiling water bath for about 5 to 10 minutes (liquid in bath should preferably not be above that in tube).
- (4) A pink color at the moistened portion of the plug indicates indol. (A blue color indicates skatol.)

5. KOVAC'S TEST

a. Reagent required:

Amyl alcohol	75 c.c.
HCl (concentrated)	25 c.c.
Paradimethylaminobenzaldehyde (Eastman)	5 g.

b. Technique:

Add 0.2 to 0.4 c.c. of the reagent to 5 c.c. of test material, shake.

A positive test is indicated by a deep red layer on the surface.

B. Tests for Ammonia**1. NESSLER'S METHOD****a. Preparation of reagent:**

(1) Dissolve 62.5 grams potassium iodide in 250 c.c. of distilled water.

(2) To 240 c.c. of this potassium iodide solution add gradually (with constant stirring) a cold saturated solution of mercuric chloride until a bright, permanent precipitate forms.

(3) Add the remaining 10 c.c. of potassium iodide and then slowly more mercuric chloride until a slight red precipitate is formed.

(4) Add to the above solution a solution made by dissolving 150 grams of potassium hydroxide in 150 c.c. of distilled water. (Solutions must be cool.)

(5) Dilute to one liter with distilled water. Allow to stand for one week and decant the supernatant fluid for use.

b. Test: The test consists of the addition of one drop of this reagent for every cubic centimeter of culture medium. Ammonia is indicated by the formation of a yellow to orange color.

2. THOMAS' METHOD**a. Preparation of reagent:**

(1) 5% solution of phenol.

(2) Sodium hypochlorite solution containing 1% available chlorine. (The solution should be so adjusted that 1 c.c. will neutralize 2.86 c.c. of N/10 sodium thiosulphate. N/10 sodium thiosulphate contains 24.8 grams per liter.)

b. Test: Dilute 0.2 to 1 c.c. of the culture to 8 c.c., then add 1 c.c. of phenol and 1 c.c. of the hypochlorite solution. Let stand for half an hour. A blue color indicates ammonia or amines.

C. Test for Nitrites**a. Reagents required:****SOLUTION A**

Sulphanilic acid.....	8 g.
5N Acetic acid (1 part glacial acetic acid to 2.5 parts water).....	1000 c.c.
or	
Dilute H_2SO_4 (1 part concentrated acid to 20 parts of water).....	1000 c.c.

SOLUTION B ¹

σ -naphthalamine.....	5 g.
5N Acetic acid.....	1000 c.c.
or	
Very dilute H_2SO_4 (1 part concentrated acid in 125 parts of water).....	1000 c.c.

The solutions are kept separate and added in equal quantities to the test medium. A red color indicates nitrites. If desired the solutions may be mixed in equal quantities before using.

D. Tests for Acidity**(Preparation of Indicators)****a. Phenolphthalein Indicator**

Phenolphthalein.....	0.5 g.
50% alcohol (neutral).....	1000.0 c.c.

(In preparing the 50% alcohol, freshly boiled and cooled distilled water should be employed.)

This indicator is red in alkaline and colorless in acid solutions; its range is pH 8.0 to 9.6.

b. Andrade's Indicator

Acid fuchsin (0.5%).....	100 c.c.
Sodium hydroxide (N/1).....	16 c.c.

(Allow to stand over night before using.)

This indicator may be employed in the culture medium. It withstands sterilization and is very stable toward bacterial de-

¹ 6 c.c. of dimethyl- σ -naphthalamine in one liter 5N acetic acid produces a much more stable test reagent and reaction.

composition. It is red in acid solution and colorless in alkaline when employed in concentrations of 1/2 to 1% in culture media.

c. *Sulphonphthalein Indicators*

(For use in culture media)

- | | |
|-----------------------|--|
| 1. Phenol red | { To indicate development of alkalinity beyond neutrality. |
| 2. Cresol red | |
| 3. Brom cresol purple | { To indicate development of acidity beyond neutrality. |
| 4. Brom phenol red | |

For use in culture media the above indicators should be prepared in concentrations of 1.6% in alcohol, and 1 c.c. of the alcoholic solution added per liter of medium.

5. Brom thymol blue; for changes on either side of neutrality. Add 2 c.c. of 2% alcoholic solution per liter of media.

d. *Mixed Indicators*

Where two indicators have useful ranges in opposite directions from and terminating at or near the neutral point they may be employed together in a medium to show development of either acid or alkali. Several such combinations follow:

1. { Phenol red; 1 c.c. of 1% solution per liter.
China blue; 2.5 c.c. of 1% solution per liter.
2. { Brom cresol purple; 1 c.c. saturated aqueous solution per liter.
Cresol red; 1 c.c. saturated aqueous solution per liter.
3. { China blue; 10 c.c. 0.5% aqueous solution per liter.
Rosolic acid; 10 c.c. 1% alcoholic (90%) solution per liter.

e. *H⁺ Ion Indicators*

Stock concentrated solutions may be prepared by grinding the dry stain with appropriate quantities of alkali as indicated in table on page 284.

For use dilute with distilled water to give appropriate concentration (0.02% to 0.04%) as indicated in Exercise 7.

Methyl red is prepared by dissolving 0.1 g. in 300 c.c. alcohol, then diluting with distilled water to 500 c.c.

PREPARATION OF STOCK SOLUTIONS OF H⁺ ION INDICATORS

INDICATOR	COMMON NAME	GRAMS	N/20 NaOH c.c.	WATER c.c.	CONCENTRATION FOR USE
Tetra-bromo-phenol-sulphon-phthalein	Brom phenol blue	0.1	3.0	22.0	0.04%
Tetra-bromo-meta-cresol sulphon-phthalein	Brom cresol green	0.1	2.9	22.1	0.04%
Di-bromo-cresol-sulphon-phthalein	Brom cresol purple	0.1	3.7	21.3	0.04%
Di-bromo-thymol-sulphon-phthalein	Brom thymol blue	0.1	3.2	21.8	0.04%
Phenol-sulphon-phthalein	Phenol red	0.1	5.7	19.3	0.02%
Cresol-sulphon-phthalein	Cresol red	0.1	5.3	19.7	0.02%
Thymol-sulphon-phthalein	Thymol blue	0.1	4.3	21.7	0.04%

E. Tests for Acetyl Methyl Carbinol

1. *Voges-Proskauer Test.* Add an equal volume of 10% NaOH or KOH to the test medium and let stand exposed to the air. A positive reaction is indicated by development of an eosine or pink coloration on surface in 1 to 24 hours.
2. *Werkman's Test.* Add an equal volume of 10% NaOH to about 5 c.c. of the test culture to which has been added 2 or 3 drops of 2% FeCl₃. Reaction more rapid than above.
3. *O'Meara's Test (Modified).* Add an equal volume of 40% KOH containing 0.3% creatin to the test medium. Eosin or pink coloration develops in 15 minutes to a few hours.

APPENDIX D

GLOSSARY OF DESCRIPTIVE TERMS

Adherent, applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; **strictly aerobic**, growing only in the presence of free oxygen.

Agglutinin, an antibody having the power of clumping suspensions of bacteria.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic**, growing only in the absence of free oxygen; **facultative anaerobic**, growing both in presence and in absence of free oxygen.

Antibody, a specific substance produced by an animal in response to the introduction of an antigen.

Antigen, a substance, which, when introduced into an animal body, stimulates the animal to produce specific bodies that react or unite with the substance introduced.

Antitoxin, an antibody having the power of uniting with or destroying a toxic substance.

Arborescent, branched, tree-like growth.

Aseptically, without permitting bacterial contamination.

Autotrophic, able to grow in absence of organic matter.

Bacteriostasis, preventing bacterial growth, but without killing the bacteria.

Beaded (in stab or stroke culture) disjointed or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Capsule, a gelatinous envelope surrounding the cell membrane of some kinds of bacteria.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Ciliate, having fine, hair-like extensions, resembling cilia, sometimes not visible to the naked eye.

Clavate, club-shaped.

Coagulation, the separation of casein from whey in milk.

Compact, refers to sediment in the form of a single, fairly tenacious mass.

- Complement**, a non-specific enzyme-like substance; destroyed if subjected to heat (56° C. or over) which occurs in blood-serum, and is necessary in conjunction with a specific antibody, in order to bring about cytolysis.
- Concentrically ringed**, marked with rings, one inside the other.
- Contoured**, an irregular, smoothly undulating surface, like that of a relief map.
- Convex**, surface growth the segment of a sphere.
- Crateriform**, a saucer-shaped liquefaction of the medium.
- Cuneate**, wedge-shaped.
- Curled**, composed of parallel chains in wavy strands, as in *anthrax* colonies.
- Cytolysin**, an antibody causing cytolysis.
- Cytolysis**, a dissolving action on cells.
- Diastatic action**, conversion of starch into simpler carbohydrates, such as dextrans or sugars, by means of diastase.
- Echinulate**, a growth along line of inoculation with toothed or pointed margins.
- Effuse**, growth thin, veily, unusually spreading.
- Endospores**, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis*.
- Endotoxin**, a toxic substance produced within a microorganism and not excreted.
- Entire**, with an even margin.
- Erose**, border irregularly toothed.
- Excentric**, slightly to one side of the center, between the positions denoted central and subterminal.
- Exotoxin**, a toxic substance excreted by a microorganism.
- Facultative anaerobe**, growing both in presence and absence of air.
- Filamentous**, growth composed of long, irregular placed or interwoven threads.
- Filaments**, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented to be distinguished from chains (q.v.) by the absence of constrictions between the segments.
- Filiform**, in stroke or stab cultures, a uniform growth along line of inoculation.
- Flaky**, refers to sediment in the form of numerous separate flakes.
- Flocculent**, containing small adherent masses of bacteria of various shapes floating in the culture fluid.
- Fluorescent**, having one color by transmitted light and another by reflected light.
- Granular**, composed of small granules.
- Hemolysin**, an antibody causing hemolysis.
- Hemolysis**, a dissolving action on red blood corpuscles.
- Immune serum**, an animal fluid containing an antibody.

Infundibuliform, in form of a funnel or inverted cone.

Intraperitoneal, within the peritoneum.

Intravenous, within a vein.

Iridescent, exhibiting changing rainbow colors in reflected light.

Lobate, having the margin deeply undulate, producing lobes (see undulate).

Luminous, glowing in the dark, phosphorescent.

Maximum temperature, temperature above which growth does not take place.

Membranous, growth thin, coherent, like a membrane.

Minimum temperature, temperature below which growth does not take place.

Mycelioid, colonies having the radiately filamentous appearance of mold colonies.

Napiform, liquefaction in form of turnip.

Opalescent, resembling the color of an opal.

Opaque, not allowing light to pass through.

Optimum temperature, temperature at which growth is most rapid.

Papillate, growth beset with small nipple-like processes.

Parasitic, deriving its nourishment from some living animal or plant upon which it lives and which acts as host; not necessarily pathogenic.

Pathogenic, not only parasitic but also causing disease to the host.

Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.

Peritrichic, covered with flagella over the entire surface.

Persistent, lasting many weeks or months.

Plumose, a fleecy or feathery growth.

Polar, at the end or pole of the bacterial cell.

Precipitin, an antibody having the power to precipitate soluble proteins.

Pulvinate, decidedly convex, in the form of a cushion.

Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.

Radiate, showing ray-structure.

Raised, growth thick, with abrupt or terraced edges.

Rapid, developing in twenty-four to forty-eight hours.

Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.

Rennet curd, coagulation of milk due to rennet or rennet-like enzymes, distinguished from acid curd by the absence of acid.

Rhizoid, growth of an irregular branched or root-like character, as in *B. mycoides*.

Ring, growth at the upper margin of a liquid culture, adhering to the glass.

Rugose, wrinkled.

Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.

Saprophytic, unable to grow in the absence of organic matter, i.e., not autotrophic, but not parasitic, as a living host is unnecessary.

Slow, requiring five or six days for development.

Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called clostridia.

Sporangia, cells containing endospores.

Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.

Strict aerobe, growing only in the presence of free oxygen.

Strict anaerobe, growing only in the absence of free oxygen.

Subcutaneous, under the skin.

Subterminal, situated toward the end of the cell but not at the extreme end, i.e., between the positions denoted excentric and terminal.

Thermophilic, growing best at high temperatures, i.e., 50° C. or over.

Transient, lasting a few days.

Translucent, allowing light to pass through without allowing complete visibility of objects seen through the substance in question.

Trituration, thorough grinding in a mortar.

Truncate, ends abrupt, square.

Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.

Umbonate, having a button-like, raised center.

Undulate, border wavy, with shallow sinuses.

Villous, having short, thick, hair-like processes on the surface, intermediate in meaning between papillate and filamentous.

Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

